A COMPARISON OF THE EFFECTS OF XENOBIOTICS ON HEPATIC HAEM METABOLISM

by

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PUBLICATIONS

Portions of the work reported in this thesis have been published with the approval of my supervisor, Prof. K.M. Ivanetich:

1. Fluroxene: Its effect on heme biosynthesis and degradation. M.R. Ziman, J.J. Bradshaw and K.M. Ivanetich, Biochem. J. 190, 571 (1980).

2. Halothane: Its effect on hepatic heme metabolism. M.R. Ziman, J.J. Bradshaw and K.M. Ivanetich, Biochem. Pharmacol., submitted for publication (1983).

3. Trichloroethylene: Its effect on hepatic heme metabolism. M.R. Ziman and K.M. Ivanetich, in press (1983).

Part of the work reported in this thesis has been presented at scientific meetings:

1. Fluroxene and porphyria. K.M. Ivanetich and M.R. Ziman, Gordon Research Conference on Drug Metabolism, July 21-25, 1980, New Hampshire, USA.

2. Fluroxene: Its effect on heme metabolism. K.M. Ivanetich, J.J. Bradshaw and M.R. Ziman, Gordon Research Conference on Pyrroles, August 4-8, 1980, New Hampshire, USA.

3. Xenobiotics: Their effects on hepatic haem metabolism. M.R. Ziman and K.M. Ivanetich. S.A. Biochemical Society Meeting, January 1983, Stellenbosch, S. Africa.

4. Degradation of hepatic cytochrome P-450: The process and its consequences. K.M. Ivanetich, M.R. Ziman, J.J. Bradshaw and A.K. Costa. S.A. Biochemical Society Meeting, January 1983, Stellenbosch, S. Africa.

ABSTRACT

Hepatic microsomal cytochrome P-450 has previously been postulated to be an important factor in determining the rates of hepatic haem biosynthesis and biodegradation. The basis for this proposal is that the haem moiety of cytochrome P-450 appears to be in equilibrium between binding to apocytochrome P-450 and existing in some form in the central hepatic pool of haem concerned with the regulation of the haem metabolic pathways. Consequently, any change in the levels of hepatic cytochrome P-450 would be anticipated to affect the pathways of hepatic haem biosynthesis and biodegradation.

At the onset of this project, relatively few chemical agents were known to destroy cytochrome P-450 (either by degradation of the haem moiety of, or dissociation of the haem moiety from hepatic microsomal cytochrome P-450) and to affect hepatic haem biosynthesis and/or haem biodegradation (e.g. AIA, Cs_2 and various metals). We thus attempted to further establish the relationship between the ability of compounds to affect hepatic cytochrome P-450 and to affect hepatic haem metabolism in vivo, using the three anaesthetic agents, fluroxene, halothane and trichloroethylene. During the preparation of this thesis, several other chemicals have been found which destroy cytochrome P-450 and affect hepatic haem metabolism (e.g. norethisterone, morphine).

In addition to the above, it has been attempted to clarify the roles of the degradation of different forms of cytochrome P-450 and of the different mechanisms of destruction of cytochrome P-450 in the control of hepatic haem metabolism. The three anaesthetic agents, fluroxene,

halothane and trichloroethylene were chosen for study since they destroy cytochrome P-450 by apparently different mechanisms. Both fluroxene and trichloroethylene specifically degrade the haem moiety of different forms of cytochrome P-450, but fluroxene converts the haem moiety of cytochrome P-450 to an N-substituted porphyrin, while TCE apparently degrades the haem into uncoloured products. In contrast, halothane appears to degrade the haem of cytochrome P-450 to uncoloured products as well as to facilitate the dissociation of haem from intact cytochrome P-450.

In general, acute administration of the anaesthetic agent confirmed the relationship between the destruction of cytochrome P-450 and the control of hepatic haem metabolism. Acute administration of fluroxene, trichloroethylene and halothane caused increases in the rates of hepatic haem biosynthesis and/or haem biodegradation. The suggestion that the effects of these compounds on hepatic haem metabolism were due to their ability to destroy cytochrome P-450 was supported by the observation that 2,2,2-trifluoroethyl ethyl ether (TFEE) and tetrachloroethylene, which are analogues of the anaesthetics, and do not or only slightly affect the levels of hepatic cytochrome P-450, had little or no effect on hepatic haem metabolism *in vivo*.

The ability of the three compounds to destroy cytochrome P-450 by different mechanisms is apparently reflected in the details of their effects on hepatic haem metabolism. A single injection of fluroxene causes the induction of both hepatic haem biosynthesis and biodegradation, whereas acute trichloroethylene treatment results in an increase in hepatic haem biodegradation, but little or no change in hepatic haem biosynthesis.

In contrast, halothane, which appears to cause both the degradation and the dissociation of cytochrome P-450, resulted in cyclical changes in hepatic haem biosynthesis and biodegradation in a manner which corresponded to the differing effects of this compound on cytochrome P-450.

Although the effects of acute administration of these compounds on hepatic microsomal cytochrome P-450 appeared to play an important role in determining their effects on hepatic haem metabolism, the effects of chronic administration of the anaesthetic agents on hepatic haem biosynthesis appeared to result from their ability (or not) to inhibit a particular enzyme of the haem biosynthetic pathway. Since a block in the haem biosynthetic pathway generally results in the induction of ALA-synthetase and an accumulation of haem precursors before the enzymatic block, chemicals which cause the inhibition of a particular enzyme of haem biosynthesis, result in the induction of a state of experimental porphyria in animals which may resemble a type of human genetic porphyria. Chronic administration of fluroxene and trichloroethylene resulted in the induction of experimental porphyria in animals by virtue of their ability to inhibit particular enzymes of the haem biosynthetic pathway whereas halothane did not appear to affect any specific enzyme of haem biosynthesis and did not induce an experimental porphyria in animals but caused cyclical changes in hepatic haem metabolism.

Chronic fluroxene administration resulted in the inhibition of the haem biosynthetic enzyme, uroporphyrinogen synthetase, and induced an experimental porphyria in animals which closely resembled Acute Intermittent Porphyria (AIP). Chronic trichloroethylene treatment appeared to affect the enzyme uroporphyrinogen decarboxylase and induced an

experimental porphyria which resembled human Porphyria Cutanea Tarda (PCT). It is therefore proposed that chronic administration of fluroxene and trichloroethylene may be useful as convenient model systems for studying the human genetic porphyrias.

From the results presented in this thesis, it is clear that the effects of chemicals on hepatic microsomal cytochrome P-450 and hepatic haem metabolism are extremely varied. Several factors appear to affect the relationship between the ability of a compound to destroy cytochrome P-450 and its ability to affect haem metabolism. It is therefore not possible at this time to predict the detailed effects of a drug on hepatic haem metabolism. However, it is hoped that this study has helped to substantiate the proposal that chemicals which destroy hepatic microsomal cytochrome P-450, will affect hepatic haem metabolism *in vivo* and to elucidate, in part, the details of this interaction.

LIST OF ABBREVIATIONS

A	: Absorbance
AIA	: Allyl-iso-propylacetamide
AIP	: Acute Intermittent Porphyria
ALA	: δ-Aminolevulinic acid
DDC	: Dicarbethoxydihydrocollidine
EDTA	: Ethylene diamine tetraacetic acid
HCP	: Hereditary Coproporphyria
HPLC	: High pressure liquid chromatography
hrs	: hours
MC	: 3-Methylcholanthrene
mic.	: microsomal
min	: minutes
NADH	: Nicotinamide adenine dinucleotide (reduced)
NADP	: Nicotinamide adenine dinucleotide phosphate
NADPH	: Nicotinamide adenine dinucleotide phosphate (reduced)
NMR	: Nuclear magnetic resonance
PB	: Phenobarbital
PBG	: Porphobilinogen
PCN	: Pregnenolone 16α-carbonitrile
PCT	: Porphyria Cutanea Tarda
S.D.	: Standard deviation
TCA	: Trichloroacetic acid
TCE	: Trichloroethylene
TFE	: 2,2,2-Trifluoroethanol
TFEE	: 2,2,2-Trifluoroethyl ethyl ether
TLC	: Thin layer chromatography
UDPGA	: Uridine 5'-diphosphoglucuronyl
VP	: Variegate Porphyria
wt.	: weight

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I INTRODUCTION

1.1. AIM

The purpose of this thesis was to investigate the effects of certain anaesthetic agents, viz. fluroxene, halothane and trichloroethylene, and their analogues, on haem biosynthesis and biodegradation. It was anticipated that some of these compounds might affect haem biosynthesis and biodegradation as a result of their metabolic activation by and subsequent destruction of cytochrome P-450 *in vivo*. Consequently, the introduction section of the thesis will consider not only the metabolism and metabolic activation of these compounds (and of xenobiotics in general) by drug metabolizing enzymes and in particular by cytochrome P-450, but it will also cover mechanisms for the degradation of cytochrome P-450. Details of the pathways for and the control of hepatic haem biosynthesis and biodegradation will be discussed as well. The possible role of cytochrome P-450 in the regulation of haem biosynthesis and in physiological disorders thereof will be considered.

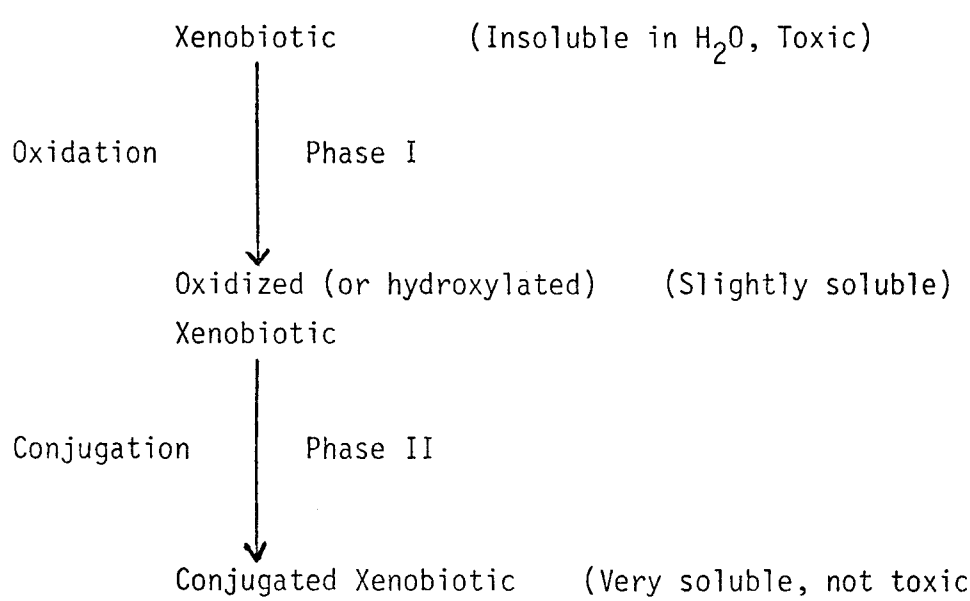
1.2. DRUG METABOLISM

Living organisms are subjected to a wide range of xenobiotics, viz. chemical compounds such as drugs, carcinogens and environmental pollutants which are foreign to the organism in as much as they are not synthesized endogenously. In mammals, the metabolic transformation of these compounds occurs primarily in the liver and is catalyzed by several groups of non-specific enzymes known collectively as drug-metabolizing enzymes (Table 1.1). Lower levels of these enzymes

are also found in other organs and tissues such as the kidney, intestine, lung, adrenal and skin (1), but for many compounds the liver is responsible for more than 95% of their metabolism.

The role of the drug metabolizing enzymes *in vivo* is essentially to protect the body against an accumulation of lipid-soluble exogenous compounds by converting them to polar hydrophilic molecules which are water-soluble and are thus more readily excreted via the kidneys into the urine than are the parent molecules. In general, the pathway by which many lipid-soluble compounds are rendered more water-soluble occurs in two distinct phases (Fig. 1.1):

FIGURE 1.1. The general pathway for the metabolism of xenobiotics.



Phase I - The oxidation of the compound by reactions such as dealkylation, deamination and hydroxylation (Table 1.1).

Phase II - The conjugation of the oxidized intermediate or of the parent compound with a small polar molecule (e.g. glucuronic acid, glutathione or sulphate) (Table 1.1) (2,3).

TABLE 1.1

HEPATIC METABOLISM OF DRUGS

Reaction	Enzyme	Primary location of enzyme
Oxidation of aliphatic and aromatic groups, N-oxidation, sulphoxidation, oxidative dealkylation, epoxidation	Cytochrome P-450	Endoplasmic reticulum
Reduction of azo-, nitro- compounds	Flavin enzymes, cytochrome P-450	Endoplasmic reticulum
Oxidation and reduction of alcohols, aldehydes and carboxylic acids	Alcohol dehydrogenase, aldehyde dehydrogenase	Cytosol
Oxidation of amines	Monoamine oxidases	Mitochondria
Hydrolysis of esters	Esterases	Cytosol and various organelles
Conjugation with glucuronic acid	UDP-glucuronyl transferases	Endoplasmic reticulum
Conjugation with glutathione	Glutathione-S-transferases	Cytosol, Endoplasmic reticulum
Conjugation with sulphate, glycine, other amino acids; acetylation and methylation	Various transferases	Cytosol and various organelles
Hydration of epoxides	Epoxide hydratase	Endoplasmic reticulum

Although the metabolism of xenobiotics is generally a detoxification process, there are a number or compounds which are converted by the drug metabolizing enzymes to more toxic, and in some cases, carcinogenic metabolites. For example, vinyl chloride is converted by enzymes of the liver to the metabolites chloroethylene oxide and/or chloroacetaldehyde (4,5), which are thought to mediate the toxicity and carcinogenicity of the parent compound; and the anaesthetic agent fluroxene is metabolized by hepatic enzymes to the toxic metabolite 2,2,2-trifluoroethanol (6).

1.2.1. Cytochrome P-450

Phase I of drug metabolism (Fig. 1.1) is catalyzed predominantly by the cytochrome P-450 enzyme system which is found within the deeper layers of the endoplasmic reticulum[+] (1,7). Cytochrome P-450 is a group of haem-containing isoenzymes[‡] which were so named because of the strong absorbance near 450 nm associated with the carbon monoxide - ferrocytochrome P-450 complex (8). This group of enzymes appears to be amongst

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[+] Homogenization of the liver tissue disrupts the endoplasmic reticulum. The resultant segments of the endoplasmic reticulum form spontaneously into small vesicles known as hepatic microsomes. These may be separated from the liver homogenate by ultracentrifugation. The microsomal fraction thus obtained from the liver is a convenient natural source of cytochrome P-450 for laboratory studies *in vitro*.

[‡] In this thesis the term 'cytochrome P-450' has been used as the general name for all of the isoenzymes of cytochrome P-450. Where required, specific terminology has been used to indicate a particular form of cytochrome P-450 - e.g. the term 'the major form of cytochrome P-450 induced by ....' refers to the isoenzyme which is induced by, for example, phenobarbital (cf. Section 1.2.1.5).

the most versatile protein catalysts known since they are capable of catalyzing the metabolism of a wide variety of drugs and other foreign compounds (9) - these include aryl hydrocarbons, polycyclic hydrocarbons, barbiturates, halogenated hydrocarbons, amphetamines and polychlorinated biphenyls. Cytochrome P-450 is also involved in the metabolism of a number of physiological substrates such as fatty acids, alkanes (10) and steroids (11).

In addition to the broad substrate specificity of cytochrome P-450, another striking feature of this group of enzymes is its ability to catalyze such a diversity of reactions; for example, both oxidative and reductive reactions are catalyzed, including an extremely wide variety of the former, such as hydroxylation, deamination, sulphoxidation, dealkylation and oxidation (Table 1.2)(12,13). The oxidative reactions catalyzed by cytochrome P-450 may all be regarded as proceeding via hydroxylation reactions even though in many cases the final product may not be hydroxylated (Table 1.2). Thus, in general, the overall reaction catalyzed by the cytochrome P-450 drug metabolizing enzymes is considered to be of the following form:

$$SH + NADPH + H^+ + O_2 \longrightarrow SOH + NADP^+ + H_2O \qquad [1]$$

where SH represents the substrate and SOH its hydroxylated product. According to equation [1], cytochrome P-450 catalyzes the oxidation of one mole of substrate and the oxidation of one mole of NADPH for each mole of molecular oxygen that is consumed. However, in experiments conducted with hepatic microsomes, the theoretical stoichiometry of 1:1:1 is not always observed due to the oxidation of NADPH by other enzymes, the oxidation of endogenous substrates by cytochrome P-450, or the uncoupling of the cytochrome P-450 enzyme system.

TABLE 1.2

REACTIONS CATALYZED BY THE CYTOCHROME P-450 DRUG METABOLIZING SYSTEM

Aromatic hydroxylation

$$CH_3CO\text{-}NH\text{-}C_6H_5 \xrightarrow{[OH]} CH_3\text{-}CO\text{-}NH\text{-}C_6H_4\text{-}OH$$

Aliphatic hydroxylation

$$R\text{-}CH_3 \xrightarrow{[OH]} R\text{-}CH_2\text{-}OH$$

N-Dealkylation

$$R\text{-}NH\text{-}CH_3 \xrightarrow{[OH]} [R\text{-}NH\text{-}CH_2OH] \longrightarrow RNH_2 + CH_2O$$

O-Dealkylation

$$R\text{-}O\text{-}CH_3 \xrightarrow{[OH]} [R\text{-}O\text{-}CH_2OH] \longrightarrow ROH + CH_2O$$

Deamination

$$R\text{-}CH(NH_2)\text{-}CH_3 \xrightarrow{[OH]} [R\text{-}C(OH)(NH_2)\text{-}CH_3] \longrightarrow R\text{-}CO\text{-}CH_3 + NH_3$$

Sulphoxidation

$$R\text{-}S\text{-}R' \xrightarrow{[OH]} [R\text{-}SOH\text{-}R']^+ \longrightarrow R\text{-}SO\text{-}R' + H^+$$

N-Oxidation

$$(CH_3)_3N \xrightarrow{[OH]} [(CH_3)_3NOH]^+ \longrightarrow (CH_3)_3NO + H^+$$

NADPH is the preferred electron donor for cytochrome P-450 dependent reactions (both oxidative and reductive). Other reductants such as NADH and ascorbate may also act as electron donors but are generally much less effective than NADPH in supporting cytochrome P-450 mediated reactions (14). For NADPH-dependent drug metabolism, electrons are passed from NADPH to cytochrome P-450 by the electron carrier NADPH-cytochrome P-450 reductase[+] (15) as shown in equation [2]:[‡]

$$\text{NADPH} \rightarrow \text{NADPH—cytochrome P-450 reductase} \rightarrow \text{cytochrome P-450} \rightarrow O_2 \qquad [2]$$

(SH → SOH)

$$\text{NADH} \rightarrow \text{NADH} - \text{cytochrome } \underline{b}_5 \text{ reductase} \rightarrow \text{cytochrome } \underline{b}_5$$

where NADH is the electron donor, NADH — cytochrome $\underline{b}_5$ reductase[+] and cytochrome $\underline{b}_5$ function as the electron carriers in the passage of electrons from NADH to cytochrome P-450 (Equation [2]) (15).

1.2.1.1. Binding of compounds to cytochrome P-450

Several drugs and other foreign compounds have been shown to bind non-covalently to hepatic cytochrome P-450 producing characteristic absorbance difference spectra (16-18). The binding of a compound to a specific binding site on the enzyme alters the environment of the haem moeity (19), giving rise to absorbance changes in the visible region of the spectrum. The most commonly observed types of difference spectra for binding to cytochrome P-450 have been identified as type I, type IR and type II (Fig. 1.2)(20)

[+] Both NADPH-cytochrome P-450 reductase and NADH-cytochrome $\underline{b}_5$ reductase are also known as cytochrome $\underline{c}$ reductases because they can transfer electrons to the non-physiological acceptor cytochrome $\underline{c}$ via cytochrome $\underline{b}_5$.

[‡] Only the last part of equation [2] differs between the oxidative and reductive reactions catalyzed by cytochrome P-450 (cf. Figs. 1.3 and 1.4).

The type I difference spectrum is a spectral manifestation of the formation of a substrate - cytochrome P-450 complex since compounds which bind to the type I binding site of cytochrome P-450 are with few exceptions metabolized by cytochrome P-450. The type I difference spectrum has been shown to arise from substrate-induced conformational changes in the protein which alter the environment of the haem moiety and alter the spin state of the haem iron atom of ferricytochrome P-450 from a low spin to a high spin state (19).

The type IR spectrum reflects the conversion of the haem iron atom of ferricytochrome P-450 from a high spin to a low spin state (20) - the opposite change in spin state to that seen with type I binding. Thus, binding of compounds to the type IR binding site of cytochrome P-450 results in the production of a spectrum which is the exact reverse of the type I difference spectrum (Fig. 1.2), but results in the exclusion of these compounds from metabolism by cytochrome P-450.

In contrast to type I and type IR spectral changes, the type II spectral change results from direct binding of the added xenobiotic to the central iron atom of the haem of cytochrome P-450 and leads to the formation of a modified ferrihaemochrome in the low spin state (Fig. 1.2) (18,20). Type II compounds compete with oxygen as a ligand for the haem iron atom of ferrocytochrome P-450 and thereby inhibit cytochrome P-450 catalyzed oxidative metabolism (21). Examples of type II binding compounds which inhibit cytochrome P-450 dependent metabolism are CO and metyrapone (22).

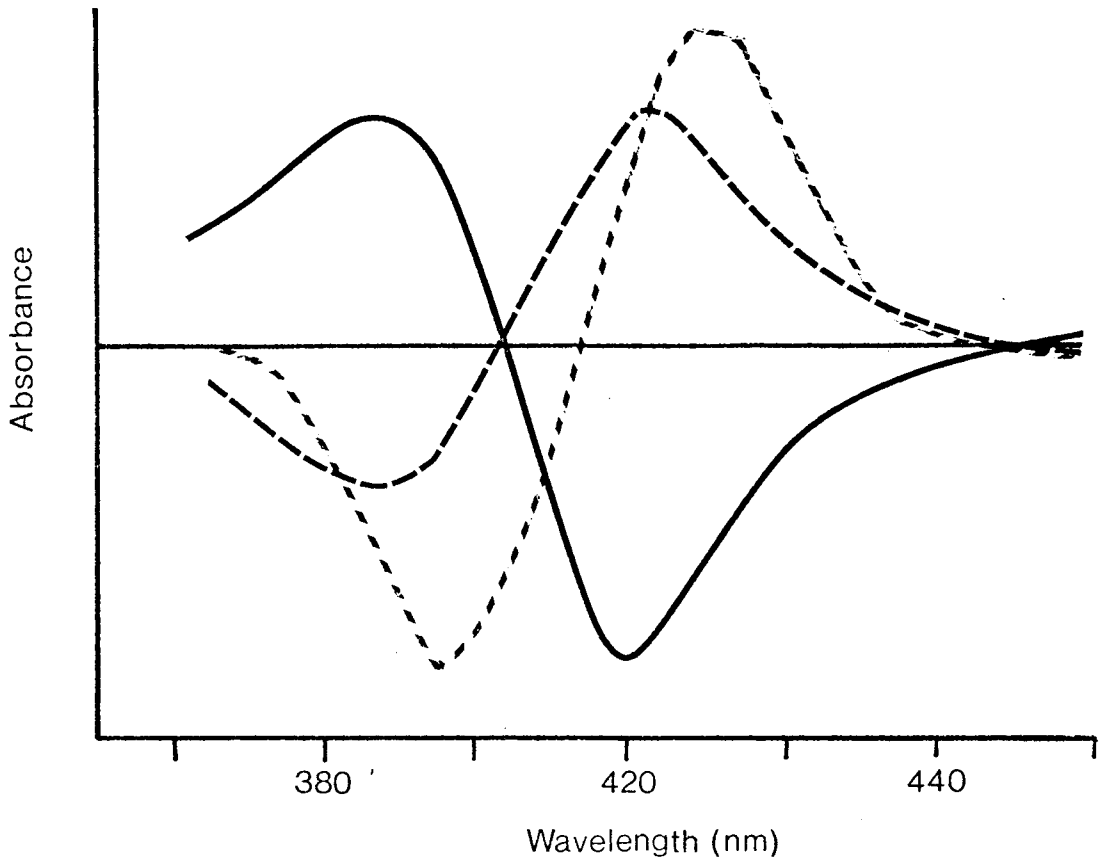


FIGURE 1.2. Difference spectral changes in hepatic microsomes. Sample cuvette contains microsomes and compound, and reference cuvette microsomes alone. Spectral changes represented: type I (——); type II (- - -); and type IR (-—-).

1.2.1.2. Cytochrome P-450 catalyzed oxidative metabolism

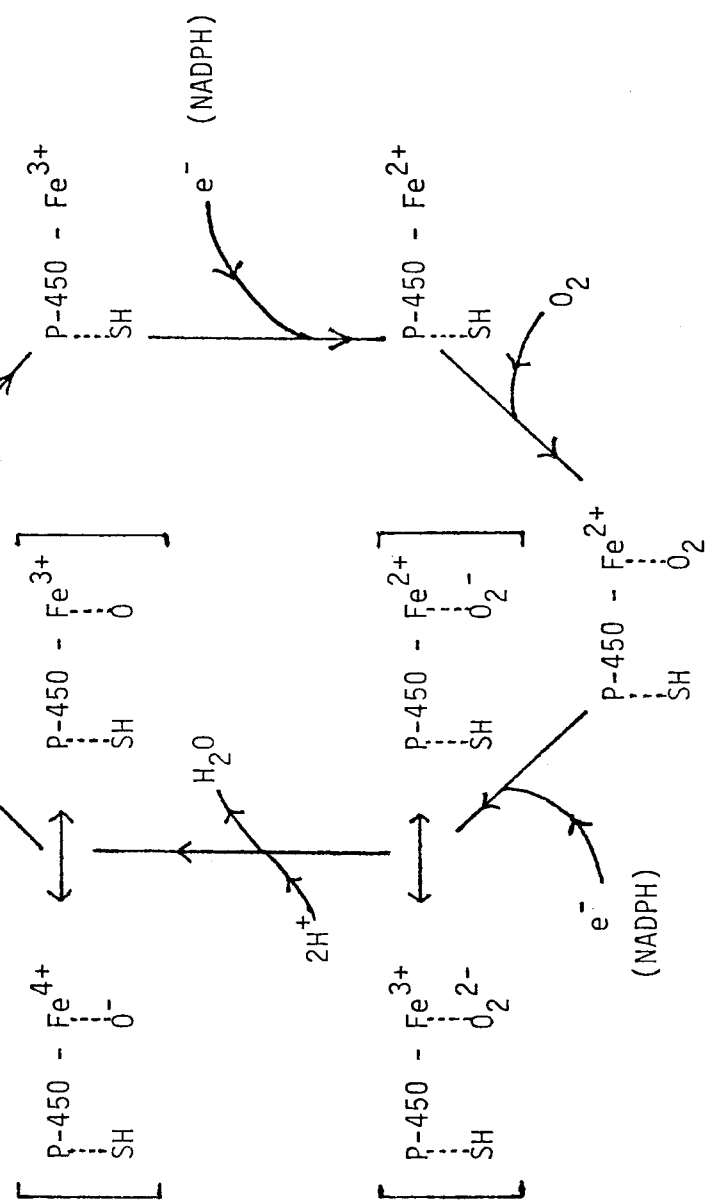
The binding of a compound to the type I binding site of cytochrome P-450 is considered to be the first step in the cytochrome P-450 dependent metabolism of a compound. The accepted mechanism for the cytochrome P-450 mediated oxidative metabolism (hydroxylation) of a compound is depicted diagrammatically in Fig. 1.3 and may be summarized as follows:

1. A substrate binds to cytochrome P-450 to form a high-spin ferri-cytochrome P-450 enzyme-substrate complex.
2. The enzyme-substrate complex undergoes a one electron reduction to a ferrocytochrome P-450 - substrate complex (23,24).
3. The ferrous enzyme-substrate complex reacts with molecular oxygen to form an oxyferrous enzyme-substrate complex (21,25).
4. This complex undergoes a second reduction stage, forming a superoxide ferrous enzyme-substrate intermediate which is a resonance form of the hydroperoxo ferric enzyme-substrate complex (26,27).
5. The hydroperoxo ferric enzyme-substrate complex decomposes by heterolytic scission of the O-O bond to produce water and a ferric enzyme-monooxygen species which is in resonance with the ferryl ion complex ($Fe^{4+}O^{-}$) (26,28).
6. The hydroxylated product is released from its binding site on cytochrome P-450, thus regenerating cytochrome P-450 in the low spin ferric state (29).

1.2.1.3. Cytochrome P-450 catalyzed reductive metabolism

Cytochrome P-450 has recently been shown to also participate in the reductive metabolism of xenobiotics such as halocarbons (30-32),

FIGURE 1.3. Mechanism of the cytochrome P-450 mediated oxidation reactions



N-oxides (33) and organic nitro and azo compounds (30). Such a reductive function of cytochrome P-450 is rather unexpected in view of the high affinity of the enzyme for molecular oxygen. However, it has been suggested that the oxygen concentration in cells may be quite low (32), especially in the centre of the liver lobules, and this allows lipophilic reducible compounds with relatively high oxidation potentials to compete effectively with oxygen for electrons at the active site of cytochrome P-450. Under these conditions, viz. low oxygen tension, the reduced form of cytochrome P-450 can transfer one or two electrons directly to the substrate, resulting in substrate reduction and the oxidation of ferrocytochrome P-450 to the ferric state (32,34,35).

Recently, much attention has been focused on the metabolism of polyhalogenated aliphatic compounds since they are widely used as solvents and often have pronounced anaesthetic properties. The metabolic transformation of these compounds in the liver results in the formation of toxic metabolites which are thought to be responsible for the liver damage promoted by these compounds in animals and man (32). There is now direct evidence that polyhalogenated alkanes can undergo oxidative and reductive metabolism catalyzed by cytochrome P-450 *in vivo* and *in vitro* (31,32,36). For most halocarbons, the extent by which reductive metabolism occurs under normal conditions, viz. high oxygen tension, is unknown but it appears to be a function of the oxygen concentration in the liver cell (32) since anaerobic conditions enhance the reductive metabolism of these compounds (32,37). However, for compounds such as carbon tetrachloride and hexachloroethane, which cannot be metabolized oxidatively due to their lack of any hydroxylatable C-H bonds, only the

reductive pathway can occur, and this has been confirmed by the identification of their exhaled volatile reductive metabolites (32).

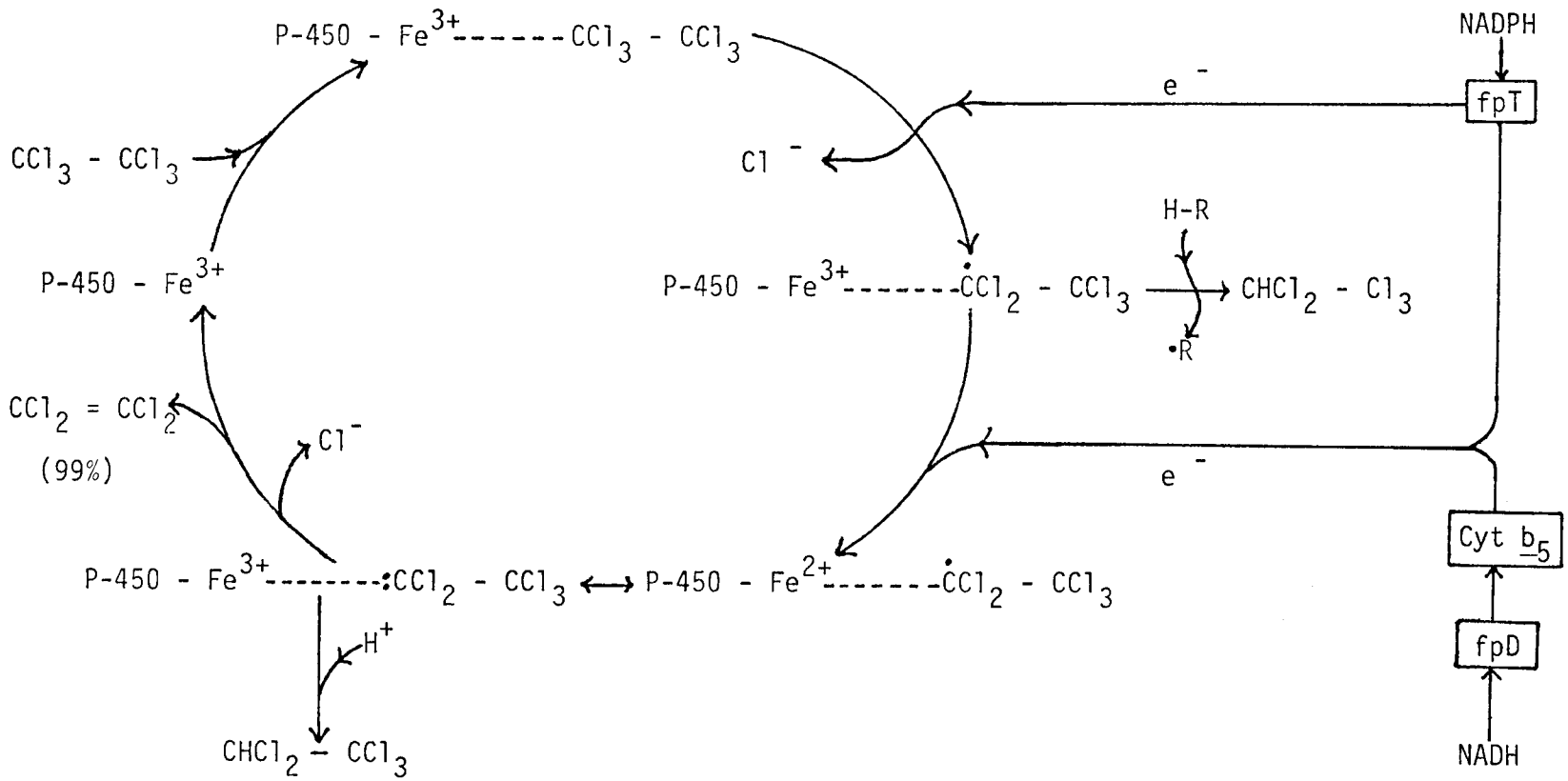
The mechanism of the cytochrome P-450 mediated reductive metabolism of these halocarbons has recently been investigated (32) and is thought to proceed via two subsequent one-electron reductions, forming first a radical and then a carbanion. The carbanion can subsequently form an alkene via β-elimination of a halide anion (32). The mechanism of the cytochrome P-450 catalyzed reductive metabolism of hexachloroethane is shown below in Fig. 1.4. Analagous reaction schemes have also been proposed for the reductive metabolism of carbon tetrachloride (38) and halothane (see Fig. 1.8) (39).

It has been proposed that the radical species thought to be generated during reductive metabolism of halogenated alkanes could initiate lipid peroxidation and thereby cause membrane damage as the crucial event of hepatotoxicity (32). Although the formation of radicals during the metabolism of some halocarbons has been demonstrated (38,40), the influence of these radical species on hepatotoxicity requires further investigation.

1.2.1.4. Multiplicity of cytochrome P-450

As mentioned earlier (Section 1.2.1), cytochrome P-450 is comprised of a group of haemoproteins. Although the debate - 'Cytochrome P-450 - one form or many' raged in the 1960's and 1970's, the evidence available presently overwhelmingly supports the existence of multiple forms of cytochrome P-450 in microsomes prepared from liver and other tissues (41). Recently, several distinct forms of cytochrome P-450 have been

FIGURE 1.4. Proposed mechanism of the reductive metabolism of hexachloroethane



P-450 - Fe^{2+} and P-450 - Fe^{3+} represent the oxidation state of the iron atom at the haem centre of cytochrome P-450; fpT is microsomal NADPH - cytochrome P-450 reductase; and fpD is microsomal NADH - cytochrome b_5 reductase. Straight lines represent electron flow whereas curved lines indicate the reaction scheme.

identified, purified and characterized (29). In rat liver, for example, using criteria such as disc gel electrophoresis, absorption maxima, immunological and catalytic properties and partial amino acid sequence, from five to seven forms of cytochrome P-450 have been identified (41,42). These isoenzymes of cytochrome P-450 differ in their apparent minimum molecular weights (29), absorbance maxima, substrate specificities and physical properties[+] (43-47). The induction of cytochrome P-450 with different agents has played an important role in the identification of cytochrome P-450 isoenzymes and will be considered in the following section (Section 1.2.1.5).

1.2.1.5. Induction[‡] of cytochrome P-450

Cytochrome P-450 is highly inducible, i.e. its activity can be greatly increased by exposure to a wide variety of xenobiotics (9, 49-51). In fact, more than 200 compounds including steroid hormones, drugs, insecticides and carcinogens are now known which stimulate drug metabolism in experimental animals and man (1, 29). Various inducing agents often selectively increase the levels of one particular form of cytochrome P-450 from the mixture of different forms of cytochrome P-450 present in hepatic microsomes (29, 41). For this reason, the use of different inducing agents has not only played a major role in establishing the

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[+] At present no systematic nomenclature is available for the multiple forms of cytochrome P-450. The nomenclature presently used by various laboratories to designate different forms of cytochrome P-450 is neither uniform nor based on a common rationale and several of the forms purified in one laboratory are probably identical to forms isolated by other laboratories (41).

[‡] The term induction in mammalian systems has been defined by Schimke and Doyle (48) as an increase in the ratio of the rate of protein synthesis over the rate of protein degradation.

existence of multiple forms of cytochrome P-450 but has also provided a very useful tool for the study of the role of a particular form of cytochrome P-450 in the metabolism and toxicity of xenobiotics.

Perhaps the most widely used inducing agent for cytochrome P-450 is phenobarbital. Administration of phenobarbital induces the isoenzyme termed 'the major phenobarbital-inducible form of cytochrome P-450' (Table 1.3) (41). This isoenzyme is responsible for the metabolism of a wide variety of substrates (15), including N,N-dimethylaniline, parathion, benzamphetamine and N,N-dimethylphentermine (41). Phenobarbital also enhances hepatic drug metabolism because it increases proliferation of the endoplasmic reticulum and the weight of the liver and, in addition, it increases the levels of NADPH-cytochrome c reductase, cytochrome b_5, cytochrome P-450 and phospholipid per mg of microsomal protein (29).

Administration of 3-methylcholanthrene or β-napthaflavone leads to the induction of the isoenzyme termed 'the major 3-methylcholanthrene - inducible form of cytochrome P-450' (Table 1.3)(41), which is also known as cytochrome P-448 or cytochrome P_1-450. This form of the enzyme is primarily responsible for the metabolism of polycyclic aromatic hydrocarbons such as 3,4-benzpyrene, 7-ethoxycoumarin, 7-ethoxyresofurin and zoxazolamine (41).

The 'PCN (pregnenolone 16α-carbonitrile) - inducible form of cytochrome P-450' (Table 1.3)(41) is another species of cytochrome P-450 which, on the basis of several criteria, is a different isoenzyme than either the major phenobarbital- or 3-methylcholanthrene-inducible forms (41). This isoenzyme catalyzes the N-demethylation of ethylmorphine and amino-

TABLE 1.3

DIFFERENT FORMS OF CYTOCHROME P-450 PURIFIED FROM RAT LIVER MICROSOMES (41)

Preparation	Treatment of Animals	CO Maximum	Subunit Molecular Weight	Comments
$P\text{-}450_b$	Aroclor 1254 PB	450	52,000	Major PB-inducible form
Fraction C	PB	450	52,000	Identical to $P\text{-}450_b$
Fraction B	PB	449.6	53,000	Major PB-inducible form; probably identical to $P\text{-}450_b$, although the end terminals of these two preparations have been found to be different
Peak I_b, phosphocellulose column	PB	450.7	50,000	Probably identical to $P\text{-}450_b$
P-450	PB	451	50,000	Probably identical to $P\text{-}450_b$
P-450	PB	Not available	48,500	Probably identical to $P\text{-}450_b$
$P\text{-}450_c$	Aroclor 1254 3-MC	447	56,000	Major 3-MC-inducible form
Fraction B	3-MC	447.6	56,000	Major 3-MC-inducible form; probably identical to $P\text{-}450_c$, although the end terminals of these two preparations have been found to be different.
Peak II, DE-52 column	3-MC	448	54,000	Probably identical to $P\text{-}450_c$
P-448	3-MC	448	56,500	Probably identical to $P\text{-}450_c$
H-II fraction	3,4,5,3',4'-Pentachlorobiphenyl	447	53,500	Probably identical to $P\text{-}450_c$
Peak III, phosphocellulose column	PCN	450	51,000	Major PCN-inducible form
$P\text{-}450_a$	Aroclor 1254 PB 3-MC	452	48,000	
Fraction D	PB	449.2	54,000	
50 mM phosphate fraction, CM-Sephadex	Cholestyramine	Not available	50,000	
80 mM phosphate fraction, CM-Sephadex	Cholestyramine	Not available	50,000	
Fraction A	None	450	52,200	
Fraction B	None	450.8	52,400	

*The abbreviations used are PB, phenobarbital; 3-MC, 3-methylcholanthrene; PCN, pregnenolone 16α-carbonitrile.

pyrine (41). Other well-known inducing agents include iso-saffrole, saffrole, ethanol, diethyl ether and aroclor 1254, to mention but a few.

1.2.2. Metabolism of fluroxene and TFEE

Fluroxene (2,2,2-trifluoroethyl vinyl ether) (Fig. 1.5) is a volatile anaesthetic agent which was introduced into clinical practice in 1953 (52). It was widely used, and it developed a reputation for safe clinical usage in man with no evidence of organ toxicity (53,54). More recently, however, laboratory tests showed fluroxene to be toxic and lethal to many animal species (55-60) and to man under certain circumstances (61-64). Following, and perhaps as a result of these studies, the manufacturers withdrew fluroxene from the market in 1977.

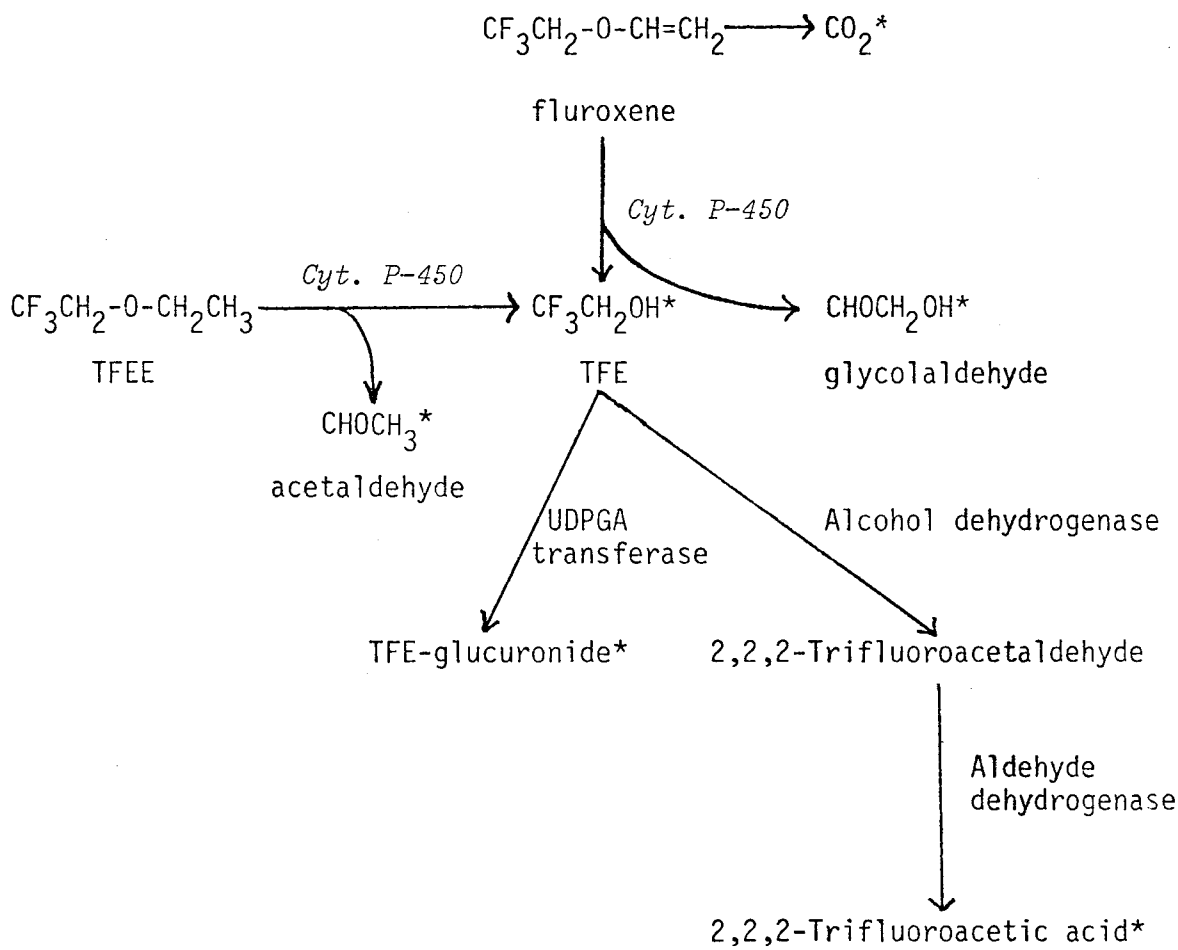
The major site of fluroxene metabolism *in vivo* is the liver, and the first step of its metabolism appears to be catalyzed by hepatic microsomal cytochrome P-450 *in vivo* and *in vitro* (65,66). From initial experiments in our laboratory using hepatic microsomes from differently induced rats (i.e. phenobarbital or 3-methylcholanthrene induced), it was concluded that the major form of cytochrome P-450 induced by phenobarbital was primarily responsible for the binding and metabolism of fluroxene, whereas the major form of cytochrome P-450 induced by 3-methylcholanthrene apparently did not bind or metabolize fluroxene (66). However, recent experiments using highly purified isoenzymes of cytochrome P-450 have shown that fluroxene is metabolized by the major form of cytochrome P-450 induced by 3-methylcholanthrene but at a much lower rate than the metabolism of fluroxene by the major phenobarbital-inducible form of cytochrome P-450 (67).

The proposed pathways of the biotransformation of fluroxene and its saturated analogue, 2,2,2-trifluoroethyl ethyl ether (TFEE), are shown in Fig. 1.5(68). The major products from the cytochrome P-450 mediated metabolism of fluroxene have been identified as 2,2,2-trifluoroethanol (TFE) and glycolaldehyde (Fig. 1.5)(58,69). TFEE, on the other hand, is metabolized by hepatic microsomal cytochrome P-450 to TFE and acetaldehyde (Fig. 1.5) (67,68). In both pathways, TFE is subsequently metabolized in vivo by microsomal UDPGA-transferase to 2,2,2-trifluoroethanol glucuronide and by cytosol alcohol dehydrogenase plus aldehyde dehydrogenase to trifluoroacetic acid (Fig. 1.5) (6,70).

Investigations into the hepatotoxicity of fluroxene and TFEE have led to the proposal that the deleterious effects of these compounds arise from their metabolism by hepatic microsomal cytochrome P-450 to the toxic metabolite TFE (Fig. 1.5) (55,59,69,71). The major evidence in support of this proposal is the observation that phenobarbital pretreatment of animals potentiates the toxic effects of both fluroxene and TFEE, presumably as a result of the phenobarbital mediated elevation in the activity of the cytochrome P-450 enzyme system causing an increase in the rate of metabolism of these compounds to TFE (70,72). Furthermore, treatment of animals with TFE results in toxic symptoms identical to those that develop after fluroxene or TFEE treatment (73).

The relative lack of toxicity of fluroxene in man as compared to animal species has been attributed to differences in the rates for the metabolism of TFE (58,74), since the major metabolites in the urine of animals after fluroxene anaesthesia are free and conjugated TFE (75), whereas in man the major urinary metabolite is the relatively innocuous

FIGURE 1.5. Metabolism of fluroxene and TFEE



* Isolated metabolites.

Abbreviations: cyt., cytochrome; TFE, 2,2,2-trifluoroethanol; TFEE, 2,2,2-trifluoroethyl ethyl ether.

compound, trifluoroacetic acid (Fig. 1.5) (76).

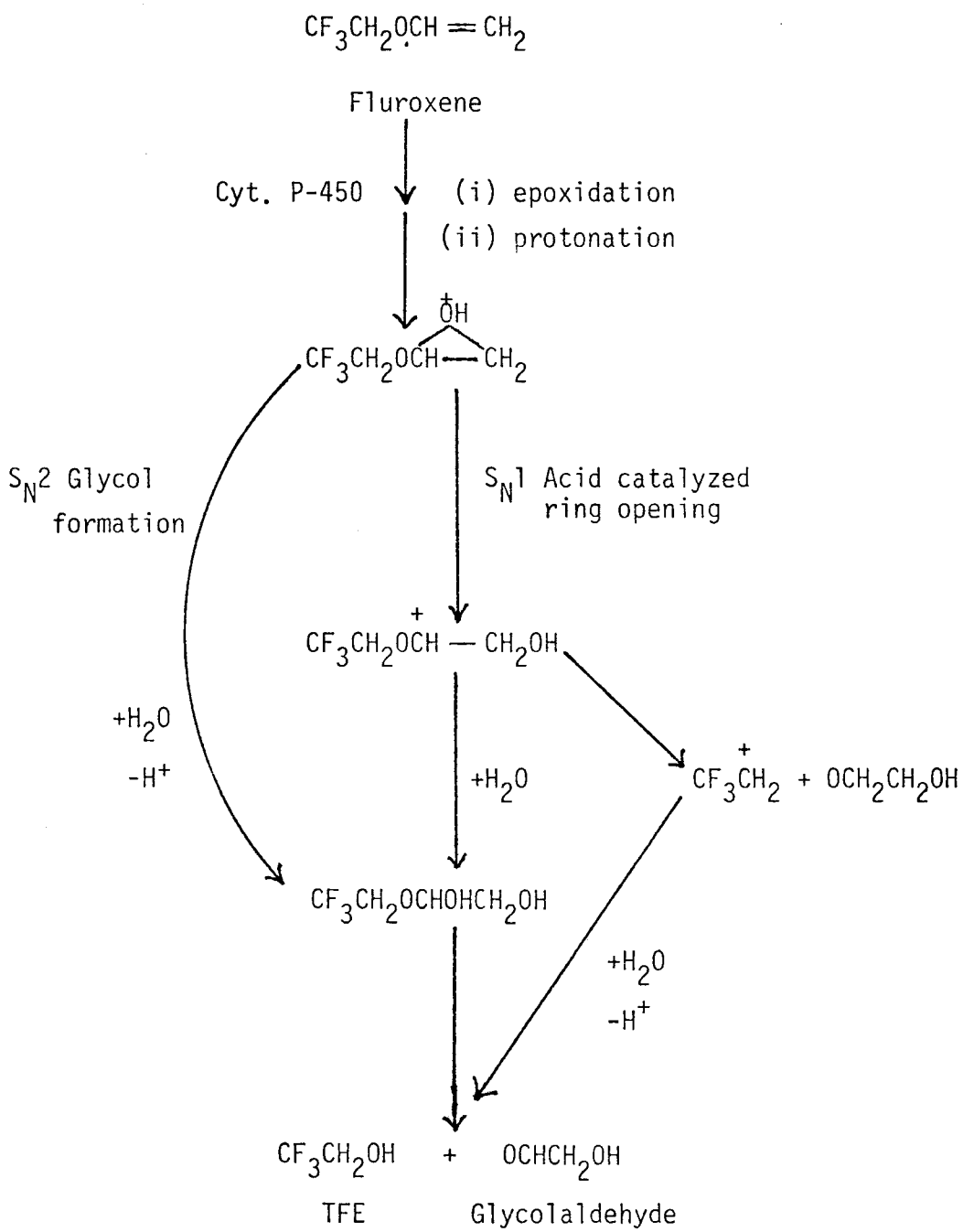
Recently, in an attempt to explain the observed metabolic products and toxic effects of fluroxene, a detailed mechanism for the conversion of fluroxene to TFE and glycolaldehyde was proposed on the basis of molecular orbital calculations (Fig. 1.6) (69). The postulated mechanism for the metabolism of fluroxene involves an initial cytochrome P-450 catalyzed epoxidation of the double bond of fluroxene (Fig. 1.6). This is followed by a non-enzymic protonation of the epoxide. The protonated epoxide can then undergo a nucleophilic attack by water via an S_N2 mechanism or an acid catalyzed hydrolysis of the epoxide (S_N1 mechanism) which would produce a glycol hemiacetal derivative (Fig. 1.6). Ether cleavage of the glycol hemiacetal derivative would generate TFE and glycolaldehyde in a stoichiometric 1:1 ratio (Fig. 1.6) (69).

1.2.3. Metabolism of halothane

Halothane ($CF_3CHBrCl$) (Figs. 1.7, 1.8) which was introduced as an anaesthetic agent in 1956 (77) is currently the most widely used volatile inhalation anaesthetic in the world, due to the relative safety of its action and its ease of administration. However, halothane anaesthesia has occasionally been associated with acute hepatic injury in man (78). Attempts to discover the cause of this toxicity have led to extensive studies of the metabolism of halothane both in animals and man (79,80).

Halothane has been shown to be metabolized to a considerable extent in humans, with as much as 24% of the administered dose of halothane being excreted as non-volatile urinary metabolites (81,82). Trifluoroacetic acid has been identified as the major urinary metabolite of halothane

FIGURE 1.6. Postulated metabolic pathways for fluroxene catalyzed by hepatic microsomal cytochrome P-450

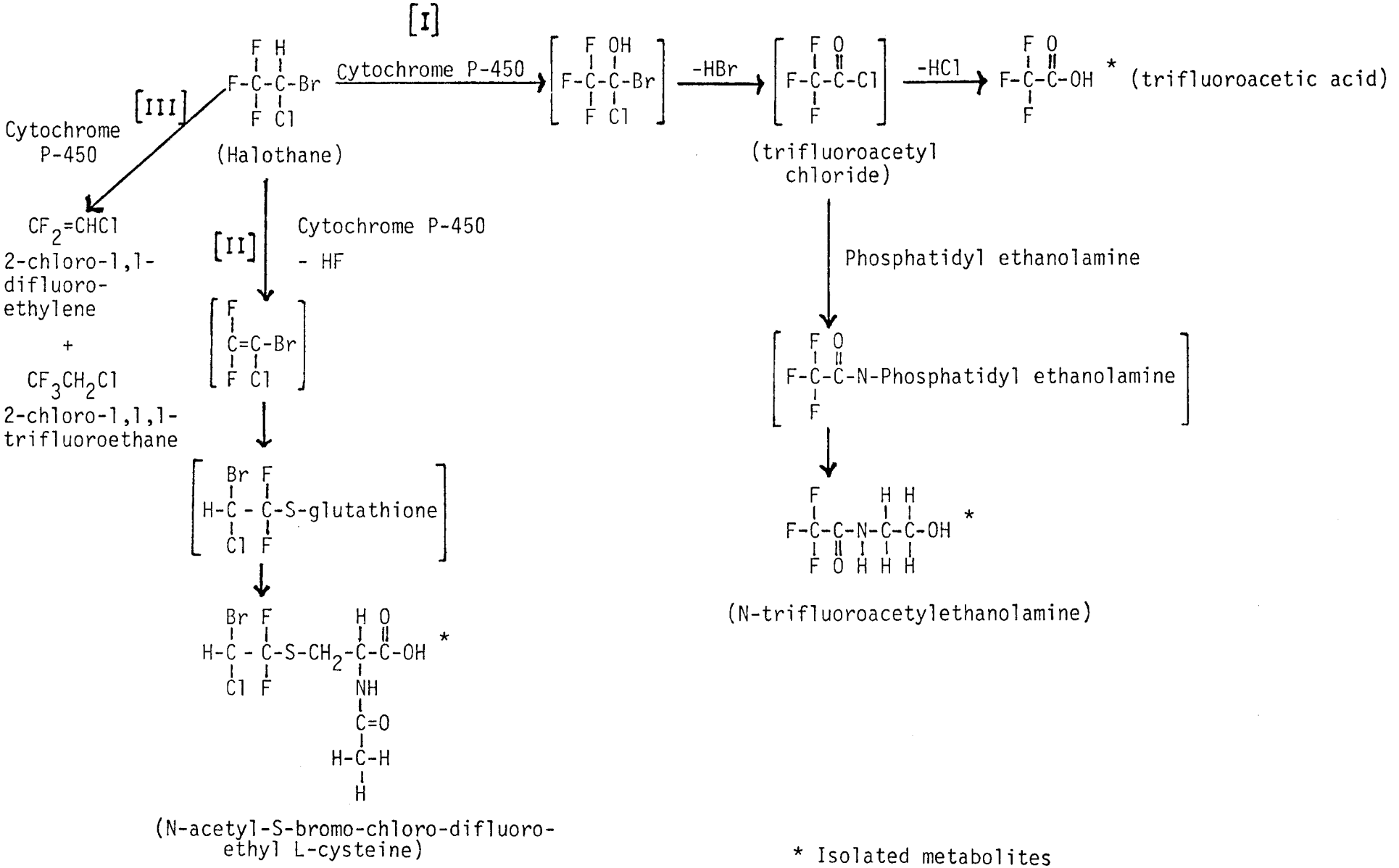


(Fig. 1.7) (82,83): Other non-volatile metabolites of halothane include N-trifluoroacetyl ethanolamine (84), N-trifluoro-2-amino-ethanol and N-acetyl-S-(2-bromo-2-chloro-1,1-difluoroethyl)-cysteine (85) as well as bromide, chloride and fluoride ions (Fig. 1.7) (86,87). Recently two novel metabolites were detected in the exhaled gases of experimental animals and man following halothane administration (34,88). These volatile metabolites were identified by Mukai et al. (88) as 2-chloro-1,1-difluoroethylene and 2-chloro-1,1,1-trifluoroethane (Figs. 1.7, 1.8).

The first and some of the subsequent steps in the biotransformation of halothane take place primarily in the liver (34). There appear to be parallel metabolic pathways for the biotransformation of halothane, viz. an oxidative pathway and one or more reductive pathways (Figs. 1.7, 1.8) (34,39,82).

The first step in the oxidative metabolism of halothane (Pathway I, Fig. 1.7) is thought to involve cytochrome P-450 catalyzed oxygen insertion on carbon number two to form 2-bromo-2-chloro-2-hydroxy-1,1,1-trifluoroethane which rapidly decomposes to trifluoroacetyl chloride. The latter compound can react spontaneously with water to form trifluoroacetic acid (34), or it can react with phosphatidyl ethanolamine to yield the non-volatile urinary metabolite, N-trifluoroacetyl ethanolamine (Fig. 1.7) (85). The cytochrome P-450 mediated oxidation of halothane (Pathway I, Fig. 1.7) is generally considered to be a detoxification reaction and is, under normal conditions (viz. normal oxygen tension), the major pathway by which halothane is metabolized in vivo (39).

FIGURE 1.7. Proposed pathways for the metabolism of halothane.

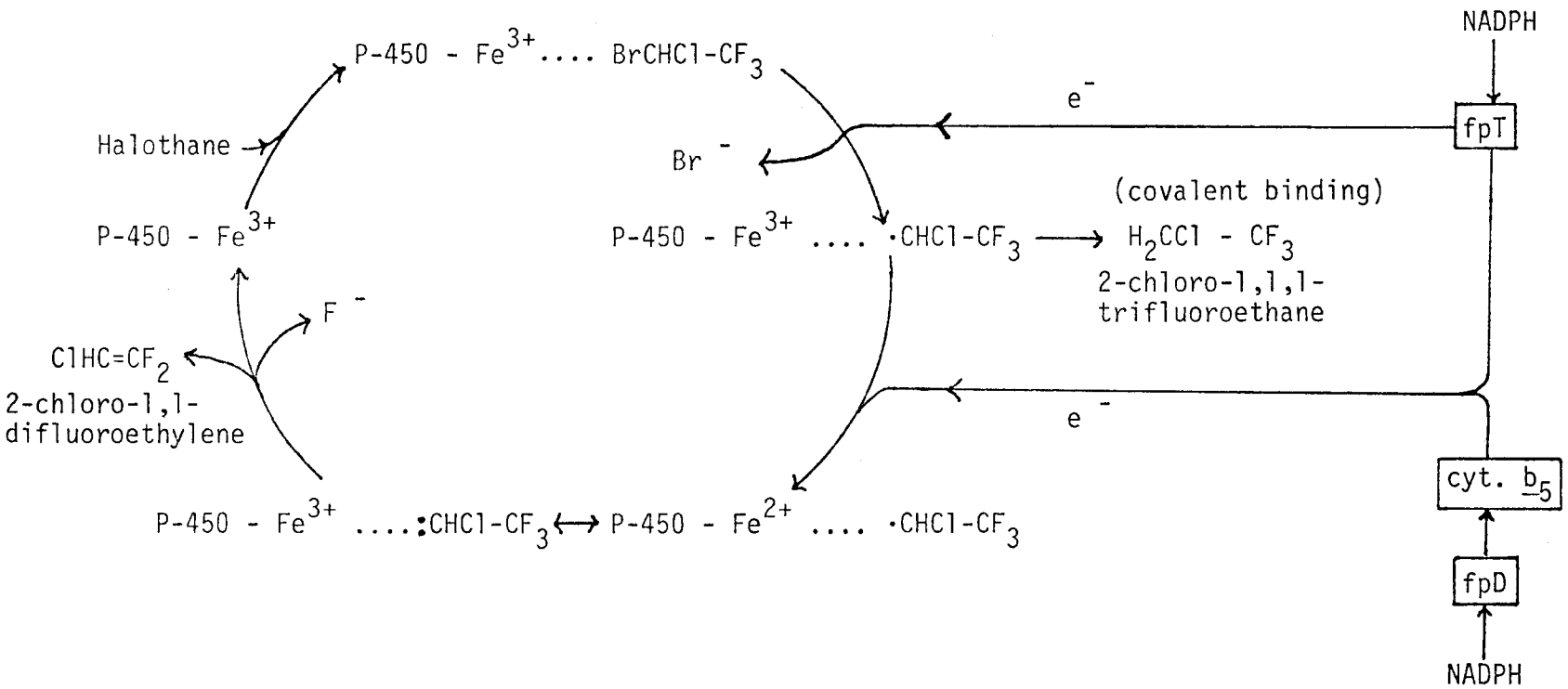


The first step of the reductive pathways of halothane metabolism (Pathways II and III, Fig. 1.7) are also catalyzed by cytochrome P-450 (34,39,89). These pathways are thought to be associated with halothane mediated hepatotoxicity in animals and humans (39). Although the importance of these pathways under normal oxygen tension is not known, they are greatly enhanced under low oxygen tension (39,90).

The reduction of halothane via Pathway II (Fig. 1.7) results in the formation of the intermediate, $F_2C=CBrCl$, a highly toxic compound which can react with cellular constituents, or with glutathione to yield N-acetyl-S(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine (Fig. 1.7) (34,85,91). Alternatively, and of far greater importance in explaining halothane toxicity, is the cytochrome P-450 mediated reduction of halothane by Pathway III (Figs. 1.7, 1.8) which leads to the formation of the volatile metabolites 2-chloro-1,1-difluoroethylene and 2-chloro-1,1,1-trifluoroethane.

A reaction scheme has been proposed for the formation of 2-chloro-1,1-difluoroethylene and 2-chloro-1,1,1-trifluoroethane which involves consecutive one electron reductions (Fig. 1.8) (39) analagous to the proposed cytochrome P-450 catalyzed reduction of haloalkanes such as carbon tetrachloride (38) and hexachloroethane (Fig. 1.4) (32). According to this mechanism (39), free radical species generated during the cytochrome P-450 mediated reduction of halothane (Fig. 1.8) may bind to microsomal proteins and unsaturated lipids and thereby initiate halothane induced liver injury.

FIGURE 1.8. Proposed mechanism for the cytochrome P-450 mediated reductive metabolism of halothane.



P-450 - Fe^{2+} and P-450 - Fe^{3+} represent the oxidation state of the iron atom at the haem centre of cytochrome P-450; fpT is microsomal NADPH-cytochrome P-450 reductase; and fpD is microsomal NADH-cytochrome $\underline{b}_5$ reductase. Straight lines represent electron flow, whereas curved lines indicate the reaction scheme.

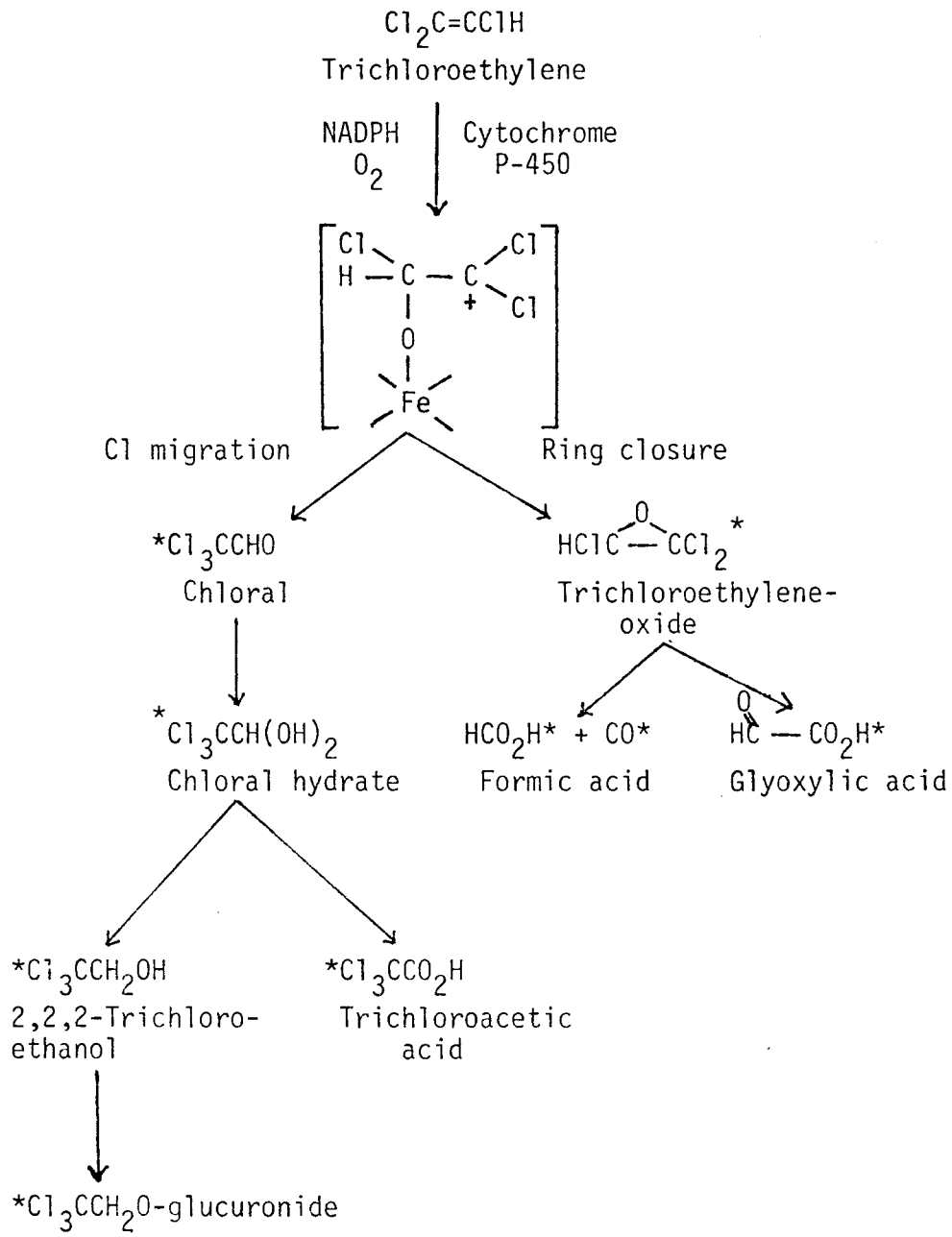
1.2.4. Metabolism of trichloroethylene and tetrachloroethylene

Trichloroethylene ($HClC=CCl_2$) (Fig. 1.9) is at present in use as a general anaesthetic agent and analgesic. It is also extensivly used in industry, primarily as a dry-cleaning solvent and metal degreasing agent. Although trichloroethylene is not considered as a potent hepatotoxin (92), exposure to trichloroethylene has been reported to result in central nervous system depression, hepatotoxicity and nephrotoxicity (93,94) and large doses of this anaesthetic agent have occasionally proved fatal to humans (93-95) and to laboratory animals (96).

Interestingly, trichloroethylene was the first inhalation anaesthetic agent found to undergo biotransformation *in vivo* (97,98). Trichloroethylene was shown to be metabolized by the liver to chloral hydrate (Fig. 1.9) (97-99) which is subsequently transformed to trichloroacetic acid and 2,2,2-trichloroethanol glucuronide , which are the major urinary metabolites of trichloroethylene (Fig. 1.9) (97,100). Other known metabolites of trichloroethylene include trichloroethylene-oxide, glyoxylic acid, formic acid and CO (Fig. 1.9) (100). Trichloroethylene is unique among inhalation anaesthetics since in addition to its own anaesthetic properties, its metabolites, chloral hydrate and 2,2,2-trichloroethanol also possess hypnotic properties which may contribute to the overall anaesthetic effect of the parent compound (97).

The first step in the oxidative metabolism of trichloroethylene to chloral has been shown to be catalyzed by hepatic microsomal cytochrome P-450 *in vivo* and *in vitro* (97,99,101-103). It was originally thought that the product of the enzyme catalyzed reaction was the epoxide, trichloroethylene-oxide which subsequently formed chloral via an internal non-

FIGURE 1.9. Proposed pathway for the metabolism of trichloroethylene.



* Isolated metabolites

[ ] - A proposed cytochrome P-450 - trichloroethylene transition state in which trichloroethylene is bound to the activated oxygen of cytochrome P-450.

enzymic rearrangement. However, it has recently been proposed that trichloroethylene-oxide is not an obligate intermediate in the metabolism of trichloroethylene to chloral in vitro (100,104). Alternatively, it has been suggested that the metabolism of trichloroethylene by cytochrome P-450 proceeds via a transition state in which trichloroethylene is bound to an activated oxygen of cytochrome P-450 (cf. Fig. 1.9) (100). This transition state may then rearrange either to chloral or trichloroethylene-oxide as shown in Fig. 1.9. Thus the cytochrome P-450 catalyzed oxidation of trichloroethylene to chloral is thought to involve chlorine migration within an oxygenated trichloroethylene - cytochrome P-450 transition state and not via trichloroethylene-oxide (Fig. 1.9) (100). Trichloroethylene-oxide is proposed to decompose to form glyoxylic acid, formic acid and CO (Fig. 1.9) (100). On the basis of the above proposals, a reaction scheme for the metabolism of trichloroethylene has been suggested and it is shown diagrammatically in Fig. 1.9 (100).

The metabolism of trichloroethylene is thought to result in the formation of reactive metabolites which bind irreversibly to proteins (105) and nucleic acids (106), thereby promoting the deleterious effects observed after trichloroethylene administration. In support of this proposal, studies of trichloroethylene metabolism have shown a direct correlation between the extent of metabolism of trichloroethylene and its hepatotoxicity (101,105,107). According to earlier reports, the reactive metabolite presumed to be responsible for these effects was trichloroethylene-oxide (101,105,108). However, Miller and Guengerich (100) suggest chloral, dichloroacetyl chloride and formyl chloride as well as trichloroethylene-oxide to be possible reactive metabolites that

might bind irreversibly to protein and nucleic acids. Further research is required to elucidate the roles of these compounds in such binding and their significance in trichloroethylene mediated hepatotoxicity.

Tetrachloroethylene ($Cl_2C=CCl_2$), a symmetrically substituted analogue of trichloroethylene, is widely used industrially as a dry-cleaning and metal degreasing agent (109,110). As a consequence of its relatively low chemical reactivity and toxicity, tetrachloroethylene is often utilized industrially in preference to trichloroethylene (110,111).

Tetrachloroethylene is primarily metabolized in the liver to trichloroacetic acid, which is the major urinary metabolite of this chlorinated ethylene in vivo (112). Hepatic microsomal cytochrome P-450 is known to catalyze the first step of the biotransformation of tetrachloroethylene (113) which is thought to proceed as follows:-

$$\underset{\text{Tetrachloro-ethylene}}{Cl_2C=CCl_2} \xrightarrow{\text{Cytochrome P-450}} \underset{\text{Tetrachloro-ethylene-oxide}}{Cl_2C(O)CCl_2} \longrightarrow Cl_3CC(=O)Cl \xrightarrow{H_2O} \underset{\text{Trichloro acetic acid}}{Cl_3CCO_2H} \quad (112,113)$$

The lower chemical reactivity and lower toxicity of tetrachloroethylene compared to that of trichloroethylene is proposed to be a consequence of the lower rate of metabolism of tetrachloroethylene (114-116) and may also be due to its conversion to less reactive metabolites[+] (113).

[+] Tetrachloroethylene-oxide is believed to be much less reactive than the epoxides of unsymmetrically substituted chlorinated ethylenes such as trichloroethylene and vinyl chloride (116,117).

1.3. DESTRUCTION OF CYTOCHROME P-450

Recent studies have revealed that the metabolism of some substances by cytochrome P-450 is accompanied by a decrease in the levels of this enzyme and consequently in diminished activities of the cytochrome P-450 drug metabolizing system (16,67,118). In many cases, this process is relatively specific for cytochrome P-450 in that other microsomal enzymes such as cytochrome b_5 and NADPH-cytochrome c reductase are affected only slightly or not at all (16, 118-121). This process is in contrast to the general devastation of proteins caused by highly toxic agents such as carbon tetrachloride which promote lipid peroxidation (120,122).

From studies of the destruction of cytochrome P-450, it has been found that the substrate must be catalytically activated by cytochrome P-450 in order for the destruction of this enzyme to occur (16,119,120). The catalytic involvement of cytochrome P-450 in its own destruction is demonstrated by the requirement for NADPH and by the inhibition of this process by potent inhibitors of cytochrome P-450 such as SKF 525A, CO and metyrapone (119,121,123). There are two clearly distinct ways in which substrates can destroy cytochrome P-450; viz [1] degradation of the haem moiety of cytochrome P-450 by its chemical modification, or [2] dissociation of the unmodified haem moiety from the apoprotein of cytochrome P-450 as a result of alteration of the structure of apocytochrome P-450 (120,123).

Degradation of the haem moiety of cytochrome P-450 is demonstrated experimentally by a quantitative decrease in the levels of hepatic microsomal haem which is equal to the observed decrease in the levels

of hepatic microsomal cytochrome P-450 (16, 119, 124), while the apoprotein of cytochrome P-450 may be unmodified (125, 126). Substrates which exhibit this degradative potential are in general unsaturated compounds such as ethylene and propyne (67, 127, 128).

Several recent experiments have shown that in some cases (but not all - cf. vinyl chloride (127)), the haem which is lost from cytochrome P-450 is converted to a haem derivative (or 'green pigment') (127, 129) thought to be formed by alkylation of the haem group of cytochrome P-450 by an activated form of the substrate (127, 129). Although several different substrates have been used to demonstrate this phenomenon, the haem derivative which is formed is, in each case, an N-substituted protoporphyrin IX, the structure of which is consistent with a mechanism involving cytochrome P-450 mediated activation of the substrate leading to covalent attachment of a portion of that substrate to a nitrogen atom of the protoporphyrin framework of prosthetic haem (127, 129-131). Degradative substrates may also be isoenzyme selective (67, 128, 132) either as a consequence of suicide inhibition or of the relative sensitivity of different isoenzymes to degradation.

The second way in which compounds can destroy cytochrome P-450 is by facilitating the dissociation of haem from the apoprotein moiety of cytochrome P-450. These compounds appear to elicit their effects by modifying the structure of apocytochrome P-450, thereby reducing the affinity of haem for the apoprotein moiety (133). In this process a loss of cytochrome P-450 is accompanied by little or no loss

of hepatic microsomal haem (16, 120) and, as a result, an increase in the amount of labile free haem[+] is observed in the livers of experimental animals after treatment with the destructive agent (16, 120, 133).

1.3.1. Destruction of cytochrome P-450 by fluroxene

The metabolism of fluroxene by hepatic microsomal cytochrome P-450 results in destruction of this enzyme *in vivo* and *in vitro* (72, 135). This destructive process appears to involve the chemical modification of the haem moiety of cytochrome P-450 since the loss of microsomal haem observed after fluroxene treatment is equivalent to the loss of cytochrome P-450 (132), while the apoprotein moiety remains unchanged (125). The unsaturated vinyl portion of the fluroxene molecule has been shown to be essential for the destructive potential of this anaesthetic because neither TFEE, nor the metabolites TFE, trifluoroacetic acid or trifluoroacetaldehyde degrade cytochrome P-450 (65, 67, 72, 135). These results also demonstrate that the fluorine substituents of fluroxene are not responsible for the observed destruction of cytochrome P-450, but it has been suggested that their presence might enhance the destruction of microsomal cytochrome P-450 *in vivo* by facilitating accessibility of this xenobiotic to hepatic microsomal cytochrome P-450 (67).

Since fluroxene itself does not degrade cytochrome P-450, it was postulated that a reactive intermediate, originally believed to be an

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[+] The appearance of a cytochrome P-420 peak (Fig. 1.10) is detectable in the microsomal preparations of animals treated with the destructive compound (123) and is thought to be representative of free haem bound to microsomal lipid (134).

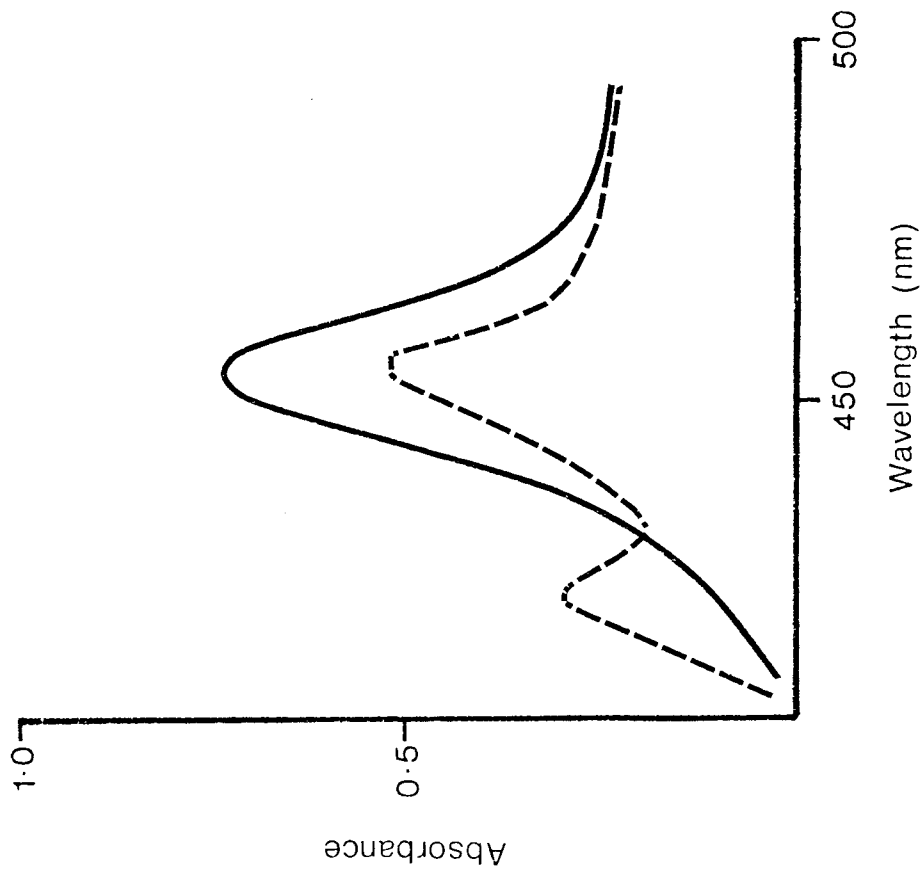


FIGURE 1.10. The appearance of cytochrome P-420 and a decrease of cytochrome P-450 in the microsomal preparations of animals treated with the destructive compound (- - -) relative to untreated animals (——). Cytochrome P-420 was so named because of the absorbance peak observed near 420 nm and is associated with haem bound to microsomal lipid (134).

epoxide arising from the metabolism of the vinyl moiety of fluroxene, was responsible for the degradation of cytochrome P-450 (135). Although attempts to isolate the epoxide of fluroxene have failed (69), it has recently been proposed that the reactive species involved in the fluroxene mediated degradation of cytochrome P-450 is not the epoxide but rather an intermediate which is formed after cytochrome P-450 catalyzed epoxidation of the double bond (69).

Recently, a mechanism for the formation of this reactive species and its subsequent degradation of cytochrome P-450 was proposed (69). It was calculated that the fluroxene epoxide (formed by cytochrome P-450 catalyzed oxidation of fluroxene (Fig. 1.6) would be stable and could diffuse away from the active site of the enzyme (69). In solution the epoxide could be protonated and decompose to products (Fig. 1.6). However, some molecules of the fluroxene epoxide could become protonated at the active site of the enzyme and the resulting carbonium ion (Fig. 1.6) could react with a nucleophilic moiety on the haem of cytochrome P-450, thus destroying the enzyme. This mechanism would explain the observation that the fluroxene mediated destruction of cytochrome P-450 is not a first order reaction (69, 136) and that the destruction process and the generation of product occur simultaneously (69).

The degradation of hepatic microsomal cytochrome P-450 by fluroxene results in the formation of a haem derivative or 'green pigment' (128, 132) which accumulates in the microsomes of fluroxene treated animals (132). This 'green pigment' was originally postulated to be an N-substituted porphyrin formed by covalent attachment of a portion of fluroxene to the haem moiety of cytochrome P-450 (137). Although

attempts were made in our laboratory to isolate and characterize this haem derivative, Kunze et al. (138) published a report showing that the exact structure of the pigment is N-(2-oxoethyl)protoporphyrin IX. This result is consistent with a mechanism in which the cytochrome P-450 catalyzed epoxidation of the unsaturated bond of fluroxene is accompanied by alkylation of the prosthetic haem of cytochrome P-450 (138).

The extent of the fluroxene mediated degradation of cytochrome P-450 is found to be enhanced by inducers of cytochrome P-450 such as phenobarbital (67, 69, 132). However, different isoenzymes of cytochrome P-450 appear to be preferentially degraded by fluroxene after different types of induction (132). In microsomes from phenobarbital induced animals, degradation appears to be non-specific with regard to different forms of cytochrome P-450, whereas in microsomes from 3-methylcholanthrene treated animals, the major form of cytochrome P-450 induced by 3-methylcholanthrene is preferentially degraded (132). Furthermore, studies with highly purified isoenzymes of cytochrome P-450 have shown that the form of cytochrome P-450 induced by β-naphthaflavone (or 3-methylcholanthrene) is more susceptible to fluroxene potentiated metabolic destruction than is the form of cytochrome P-450 induced by phenobarbital (67, 128). Thus, although the destruction of both forms of cytochrome P-450 is extensive, the relatively lower rate of metabolism of fluroxene by the form of cytochrome P-450 induced by β-naphthaflavone (or 3-methylcholanthrene) indicates that this isoenzyme of cytochrome P-450 produces less activated metabolite but is more sensitive to destruction (67).

1.3.2. Destruction of cytochrome P-450 by halothane

The metabolism of halothane by hepatic microsomal cytochrome P-450 is accompanied by a reduction in the levels of hepatic cytochrome P-450 *in vivo* and *in vitro* (16, 78, 139). This process appears to be specific for cytochrome P-450 since the levels of other hepatic microsomal proteins, such as cytochrome b_5 and NADPH-cytochrome *c* reductase, are not affected by halothane (16, 78).

Studies of the metabolism of halothane *in vitro* have shown that the loss of hepatic microsomal cytochrome P-450 exceeds the loss of hepatic microsomal haem and that the loss of cytochrome P-450 is accompanied by an increase in cytochrome P-420 (Fig. 1.10) after halothane treatment (16). These results indicate that the halothane mediated decrease in the levels of hepatic cytochrome P-450 *in vitro* appears to be partly due to the modification of the haem moiety of cytochrome P-450 and partly due to the dissociation of the haem moiety from cytochrome P-450 (presumably as a result of halothane mediated denaturation of the apoprotein) (16).

1.3.3. Destruction of cytochrome P-450 by trichloroethylene and tetrachloroethylene

Trichloroethylene metabolism has been shown to result in the degradation of hepatic microsomal cytochrome P-450 *in vivo* and *in vitro* (99, 100, 107). It would appear that trichloroethylene specifically degrades the haem moiety of hepatic microsomal cytochrome P-450 since the trichloroethylene mediated decrease in the levels of hepatic microsomal cytochrome P-450 is accompanied by an identical decrease in the levels of hepatic microsomal haem (99).

Since trichloroethylene itself does not degrade cytochrome P-450 (99), it was postulated that the epoxide, trichloroethylene-oxide (thought to be formed by cytochrome P-450 catalyzed oxidation of trichloroethylene (Fig. 1.9)) was the reactive species responsible for the degradation of the haem of cytochrome P-450. However, evidence has recently been obtained which shows that epoxide metabolites, including trichloroethylene-oxide, play at most, a minor role in the destruction of cytochrome P-450 mediated by the parent olefins (100, 127, 140). It appears likely therefore, that a reactive intermediate is generated during or after cytochrome P-450 catalyzed epoxidation of the double bond (69, 100).

Different types of inducing agents appear to affect the metabolism of trichloroethylene by hepatic microsomal cytochrome P-450 and the degradation of the haem of cytochrome P-450 by trichloroethylene to similar extents (99). Induction of cytochrome P-450 with phenobarbital enhances the metabolism of trichloroethylene as well as the trichloroethylene mediated degradation of cytochrome P-450, whereas induction of cytochrome P-450 with 3-methylcholanthrene reduces the metabolism of trichloroethylene and the trichloroethylene mediated degradation of cytochrome P-450 (99).

As opposed to trichloroethylene, tetrachloroethylene, the fully chlorinated analogue of trichloroethylene, does not affect the levels of hepatic microsomal cytochrome P-450 and haem *in vitro* (113) and only slightly decreases the levels of hepatic microsomal cytochrome P-450 *in vivo* (141). The less severe effects of tetrachloroethylene on the levels of hepatic microsomal cytochrome P-450 may reflect a lower rate

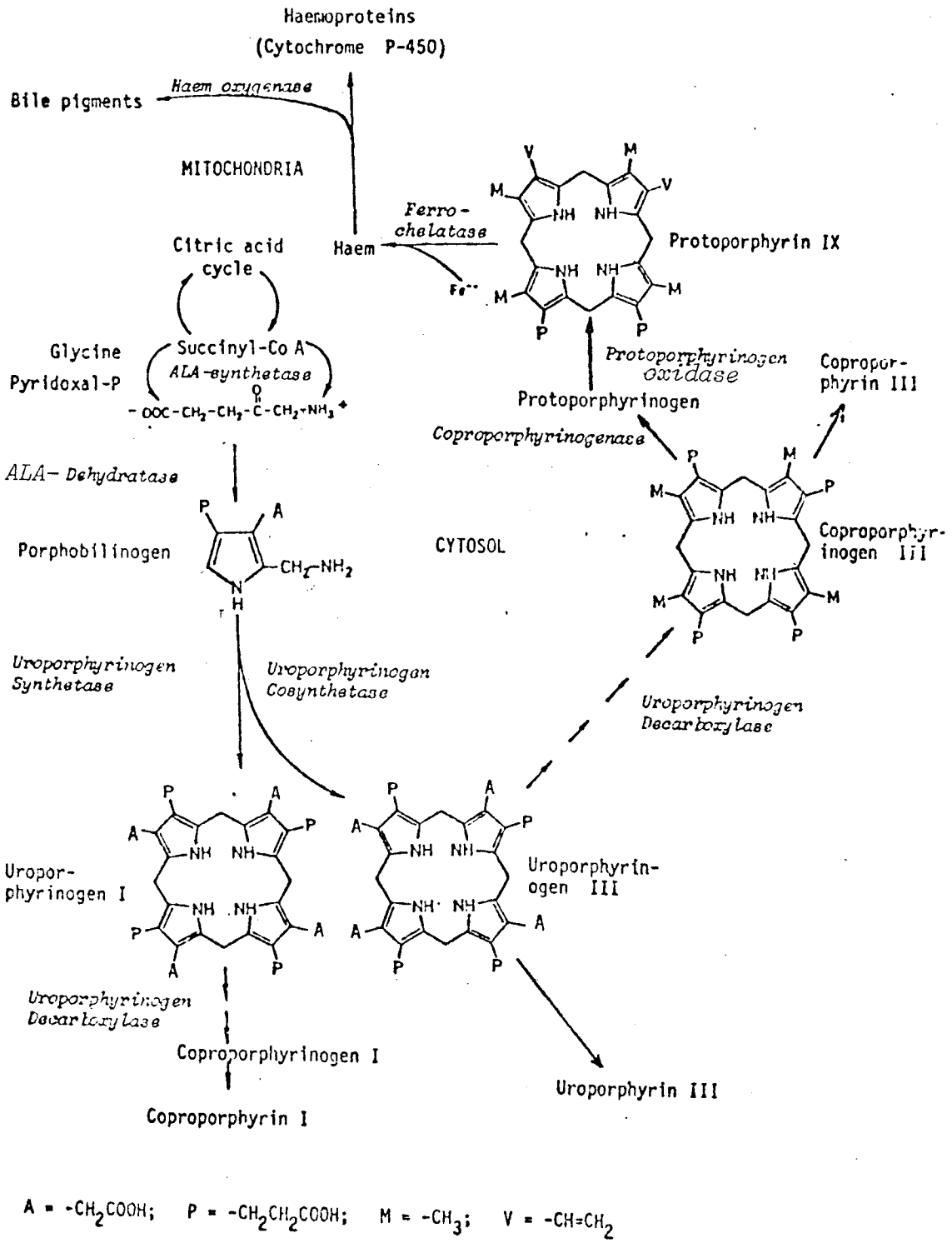
of metabolism of tetrachloroethylene and/or conversion to a less reactive metabolic intermediate than trichloroethylene (113, 117).

1.4. HAEM BIOSYNTHESIS

The biosynthesis of haem has been extensively studied (for reviews, see 142-146). The overall pathway of haem biosynthesis is outlined in Fig. 1.11 and may be summarized as follows:
The first step in the pathway is the condensation of succinyl CoA (147) and glycine (148) to form δ-aminolevulinic acid (ALA) (Fig. 1.11) (149). This reaction takes place in the mitochondria (150-152) and is catalyzed by ALA-synthetase (153) which is the rate-limiting enzyme of the haem biosynthetic pathway in normal circumstances (153, 154). ALA-synthetase is thought to exist as a dimer with a molecular weight of approximately 120 000 (155). Pyridoxal-5'-phosphate, which is an essential cofactor for this enzyme, is bound to ALA-synthetase more strongly than either of its substrates (151, 156-158). Although the major fraction of ALA-synthetase is found in the mitochondrion (150, 159, 160), some ALA-synthetase is also found in the cytosol but it is thought to be a precursor form in transit to the mitochondrion (161).

The second step of the haem biosynthetic pathway, viz. the condensation of two molecules of ALA to form the monopyrrole porphobilinogen (162), takes place in the cytoplasm (Fig. 1.11) (163). The enzyme which catalyzes this reaction, ALA-dehydratase (164, 165), consists of eight identical subunits arranged as a pair of tetramers (166). Zn^{2+} ions, which bind to the sulfydryl groups of the enzyme, are required for maximum activity (167).

FIGURE 1.11. The haem biosynthetic pathway.



A = $-CH_2COOH$; P = $-CH_2CH_2COOH$; M = $-CH_3$; V = $-CH=CH_2$

Four molecules of porphobilinogen are condensed to form the octa-carboxylic tetrapyrrole known as uroporphyrinogen (Fig. 1.11). Two different stereo-isomeric forms of uroporphyrinogen are formed during the biosynthesis of haem, viz. the type I and type III isomers (Fig. 1.11). The formation of uroporphyrinogen III is catalyzed by the action of two independent, sequentially acting enzymes known as uroporphyrinogen I synthetase and uroporphyrinogen III cosynthetase (Fig. 1.11) (143, 168-170). However, in the presence of uroporphyrinogen I synthetase alone, four molecules of porphobilinogen are converted to one molecule of uroporphyrinogen I (Fig. 1.11) (171-173), whereas uroporphyrinogen III cosynthetase alone cannot accept either porphobilinogen or uroporphyrinogen I as a substrate (170,174).

Uroporphyrinogen III, which contains 8 carboxyl groups, undergoes stepwise enzymatic decarboxylation of all four of its acetic acid side chains to produce coproporphyrinogen III (Fig. 1.11) (175, 176). It appears that a single enzyme, known as uroporphyrinogen decarboxylase, effects the four successive decarboxylations proceeding in a clockwise manner about the molecule (177-179). Uroporphyrinogen I is also decarboxylated by this enzyme to coproporphyrinogen I (Fig. 1.11), but this reaction proceeds at a slower rate than the decarboxylation of the type III isomer (178-182). The oxidation of uro- and coproporphyrinogen intermediates to their corresponding porphyrins (Figs. 1.11 and 1.12) results in the exclusion of these compounds from the haem biosynthetic pathway (180).

The conversion of coproporphyrinogen III to protoporphyrinogen IX

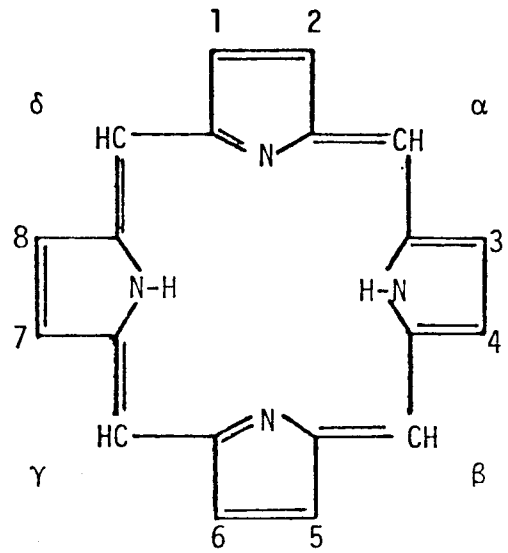
(Fig. 1.11) involves an oxidative decarboxylation of two adjacent propionate groups to yield two vinyl moieties. This process takes place in the mitochondria and is catalyzed by coproporphyrinogenase (183). This enzyme is specific for the type III isomer of coproporphyrinogen, i.e. it does not react with the type I isomer of coproporphyrinogen (184). The subsequent oxidation of protoporphyrinogen IX to protoporphyrin IX (Figs. 1.11, 1.12) is catalyzed by the enzyme protoporphyrinogen oxidase (185-187).

The final step in haem biosynthesis is the incorporation of a ferrous ion (Fe^{2+}) into protoporphyrin IX (Fig. 1.11). This reaction is catalyzed by the mitochondrial enzyme, ferrochelatase (188, 189).

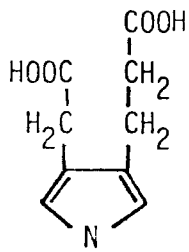
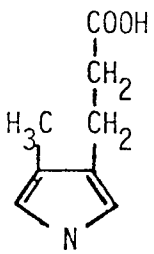
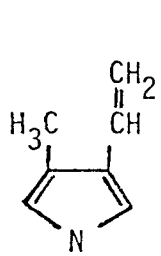
The haem thus formed combines with various apoproteins to yield the haem containing enzymes of the liver such as cytochromes, catalase, tryptophan pyrrolase and peroxidase. As can be seen in Fig. 1.11, the type I porphyrinogens are by-products of the haem biosynthetic pathway since only the type III porphyrinogens are intermediates in the biosynthesis of haem.

The major by-products of the pathway, viz. the type I porphyrins, as well as small amounts of the type III porphyrins are normally excreted in urine and faeces (190). Porphyrins with more than 4 carboxyl groups including uroporphyrin (Fig. 1.12) are predominantly excreted in the urine (191), while those with less than 4 carboxyl groups, e.g. protoporphyrin (2 carboxyl groups) (Fig. 1.12) are eliminated exclusively in the faeces (192). Coproporphyrin (4 carboxyl groups) (Fig. 1.12) appears in both urine and faeces (190). Differences in the routes of excretion of porphyrins are brought about through differences in their water solubility.

FIGURE 1.12. Porphyrin Ring System



Porphyrin nomenclature

	(X) (Y) 	(X) (Y) 	(X) (Z) 
Uroporphyrin	4	--	--
Coproporphyrin	--	4	--
Protoporphyrin	--	2	2

For 2 substituents [Type I 1, 3, 5, 7 - X; 2, 4, 6, 8 - Y

Type III 1, 3, 5, 8 - X; 2, 4, 6, 7 - Y

For 3 substituents [Type I 1, 3, 5, 7 - X; 2, 4 - Z; 6, 8 - Y

Type III 1, 3, 5, 8 - X; 2, 4 - Z; 6, 7 - Y

Substituents

acetate - CH_2CO_2H

propionate - $CH_2CH_2CO_2H$

methyl - CH_3

vinyl - $CH{=}CH_2$

In addition, small amounts of the other metabolic intermediates of haem biosynthesis, viz. ALA and porphobilinogen (193) and various type I and III porphyrinogens (Fig. 1.11) (194) are excreted in the urine of normal mammals.

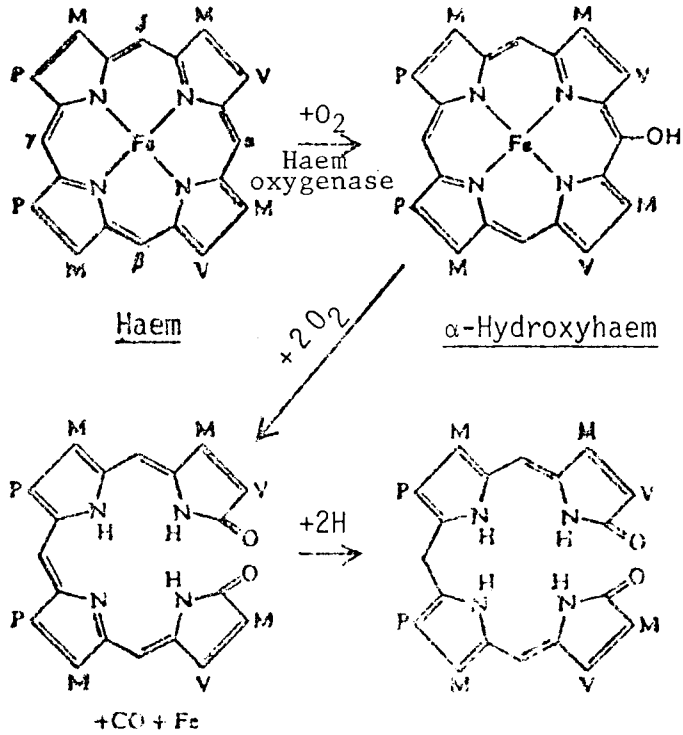
Since most aerobic cells can synthesize haem, it is likely that small amounts of porphyrins are formed in virtually all tissues of the mammalian organism. In relation to the overall extent to which haem is synthesized in the body, the amounts of porphyrins and porphyrin precursors that are excreted in normal individuals are very small, thereby attesting to the efficiency of the haem biosynthetic pathway (190,195).

1.5. HAEM BIODEGRADATION

The first step in the catabolism of haem (Figs. 1.13, 1.14) is catalyzed by the microsomal enzyme, haem oxygenase (196). The binding of haem by haem oxygenase confers a transitory haemoprotein nature to the enzyme (197). The newly formed haemoprotein can then accept reducing equivalents from NADPH via microsomal NADPH-cytochrome c reductase (198). Haem oxygenase thus functions as the terminal oxygenase in the oxidative catabolism of haem (199), with haem acting as both substrate and coenzyme (200,201).

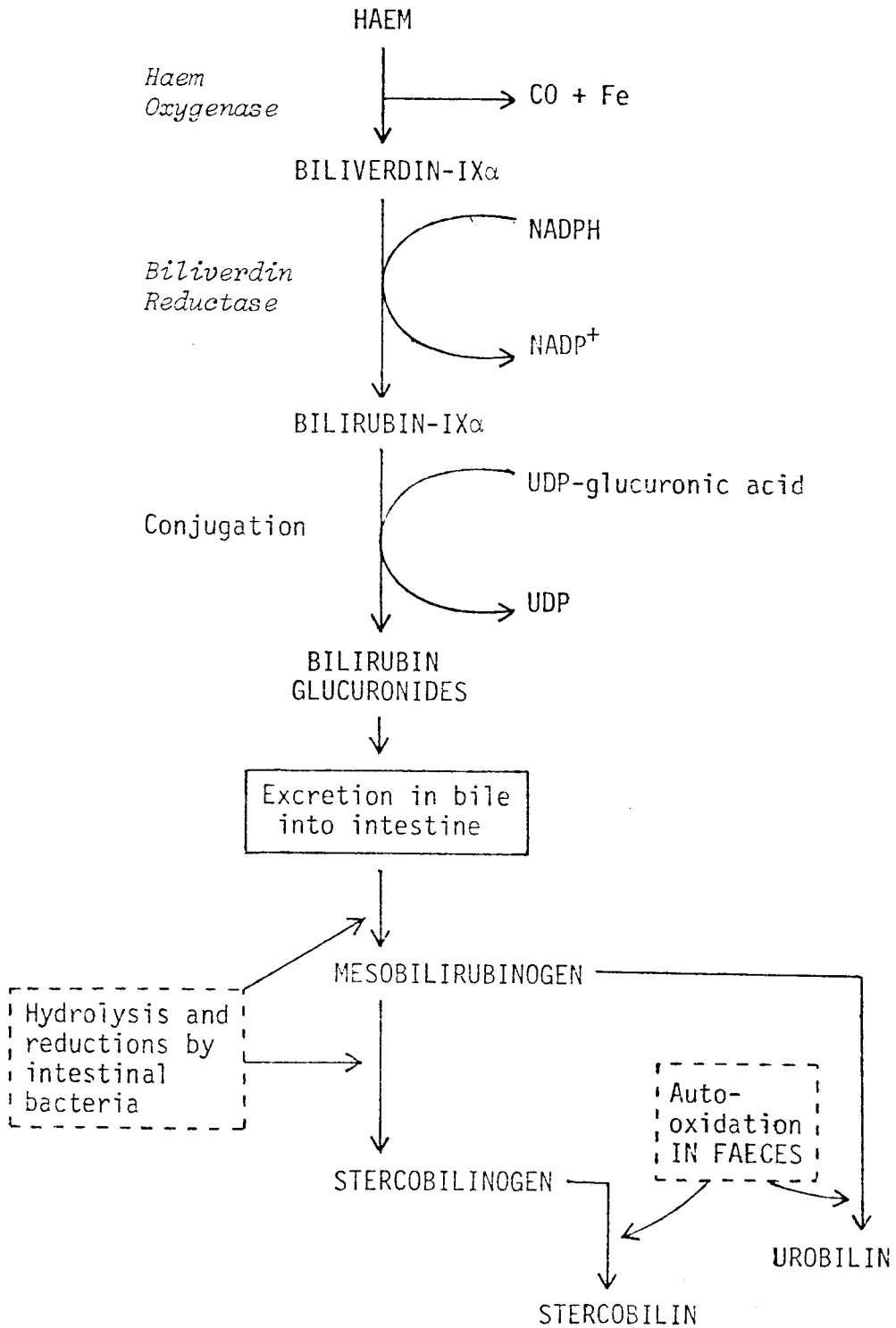
The first step in the oxidation of haem by haem oxygenase is assumed to be the hydroxylation of the α-methene carbon of protohaem, which is then followed by cleavage of the haem ring to yield biliverdin IXα (Figs. 1.13, 1.14) (200,202). The precise way in which oxygen is involved in the reaction has not yet been determined, but the formation of a haem ion-oxygen complex is thought to take place on the enzyme prior to the cleavage of the haem ring (201,203).

FIGURE 1.13. Degradation of haem to bile pigments.



M = $-CH_3$; V = $-CH{=}CH_2$; P = $-CH_2CH_2CO_2H$.

FIGURE 1.14. Haem biodegradative pathway.



UDP = uridine diphosphate

The subsequent step in the biodegradation of haem is the conversion of biliverdin IXα to bilirubin IXα (Figs. 1.13, 1.14) by biliverdin reductase, which is present in excess in liver cytosol (196, 204). Bilirubin IXα is conjugated to glucuronic acid and eventually deconjugated before it is excreted via the bile of humans and animals (Fig. 1.14) (205).

1.6. HEPATIC REGULATION OF HAEM BIOSYNTHESIS AND BIODEGRADATION

The biosynthesis of haem is regulated by the activity of the first enzyme in the haem biosynthetic pathway, ALA-synthetase*(Fig. 1.11) (150, 206). Furthermore, the amount and activity of this enzyme are regulated by haem, the end-product of the pathway, in a manner which involves repression of the synthesis of the enzyme at a transcriptional (207) or post-transcriptional level (208), and/or by a mechanism which prevents the transfer of precursor ALA-synthetase from the cytosol to the mitochondria (208, 209). Thus haem controls its own rate of synthesis by controlling the activity of ALA-synthetase. Haem also regulates the rate of its biodegradation since haem oxygenase, which catalyzes the first and rate-controlling step in the pathway for the biodegradation of haem, is inducible by haem (133).

The results of recent experiments, using isolated hepatocytes, suggest that a central pool of hepatic haem exists in the cytosol into which newly synthesized haem is incorporated and from which haem is drawn for the synthesis of hepatic haemoproteins, and for the degradation of haem to bilirubin (210, 211). It has been postulated that the size of this central 'free' haem pool regulates the activity of ALA-synthetase (211-215), and of haem oxygenase (133, 211, 215), thereby controlling

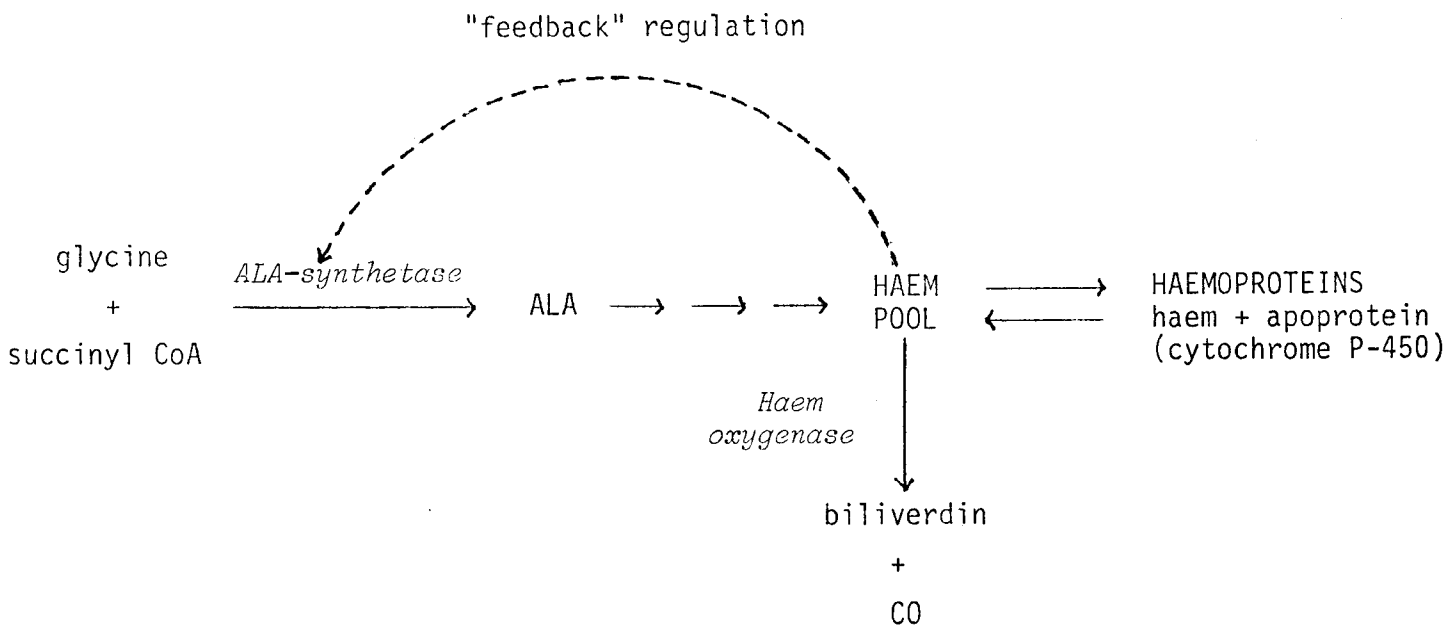
* Uroporphyrinogen synthase has been shown to act as a secondary rate controlling step in haem biosynthesis.

the rate of haem biosynthesis and haem biodegradation (Fig. 1.15).

In part, the levels of haem in the hepatic regulatory haem pool are thought to be controlled by hepatic cytochrome P-450. It has been suggested that hepatic microsomal cytochrome P-450 dissociates into haem plus apoprotein with the haem in equilibrium between the regulatory 'free' haem pool and apocytochrome P-450 (Fig. 1.15) (133, 210, 211, 216). This proposal is supported by the observation that exogenous haem is incorporated into intact apocytochrome P-450 in vivo and is thought to mix with newly synthesized haem in the 'free' haem pool prior to its incorporation into cytochrome P-450 (216-218).

In the liver, large quantities of haem are required for the synthesis of haemoproteins, particularly hepatic microsomal cytochrome P-450 (219) - more than 50% of haem synthesized in the liver is utilized in the formation of cytochrome P-450 (133, 142, 210). Thus the dissociation or degradation of haem from cytochrome P-450 should have a significant effect on the size of the regulatory haem pool and consequently exert an effect on haem biosynthesis via ALA-synthetase activity and/or haem biodegradation via an effect on the activity of haem oxygenase (Fig. 1.15). This phenomenon has been shown to occur in the presence of chemicals such as allyl-isopropylacetamide (AIA) which degrade the haem moiety of cytochrome P-450 (see Section 1.8.1.3).

FIGURE 1.15. Proposed scheme for the regulation of ALA-synthetase and haem oxygenase by hepatic haem.



1.7. PORPHYRIA

Metabolic disorders in the regulation of haem biosynthesis and biodegradation are known to occur. These disorders, which manifest themselves as a group of diseases which are known as the porphyrias, are characterized by an accumulation of the intermediates of the haem biosynthetic pathway, i.e. ALA, porphobilinogen and the porphyrins (220). Since the major sites of haem biosynthesis in mammals are the liver and the erythroid elements of bone marrow, the porphyrias are classified according to whether the bulk of excessive porphyrin production takes place in the liver or bone marrow; viz. hepatic porphyria or erythropoietic porphyria respectively (Table 1.4) (221).

Since most of the porphyrias are autosomal dominant genetic diseases, it can be shown that each of the porphyrias represents a different inborn error of metabolism in haem biosynthesis (Fig. 1.16) (221). There is, therefore, in each type of porphyria a different and characteristic pattern of excretion of haem precursors (Table 1.5) (145, 222). Because the disorders are generally hereditary, any enzyme defect would be present in all somatic cells, and indeed such defects have been found in fibroblasts, amniotic cells and peripheral blood, in addition to the liver and bone marrow (223, 224).

The activity of ALA-synthetase, the rate controlling enzyme in haem biosynthesis, is markedly raised in most varieties of human porphyria with the exception of Porphyria Cutanea Tarda, where the increase is relatively small (Table 1.4) (225). Since the activity (226) of ALA-synthetase is thought to be regulated by haem through a negative feedback control (Fig. 1.15), a genetic defect in the haem biosynthetic pathway

TABLE 1.4

CLASSIFICATION OF HUMAN PORPHYRIAS

HEPATIC PORPHYRIAS

I) GENETIC AUTOSOMAL DOMINANT PORPHYRIAS

1) Acute Intermittent Porphyria (AIP)*

2) Variegate Porphyria (VP) (S. A. Porphyria)*

3) Hereditary Coproporphyria (HCP)* (rare)

4) Porphyria Cutanea Tarda (PCT)

II) ACQUIRED PORPHYRIA

1) Porphyria Cutanea Tarda (PCT)

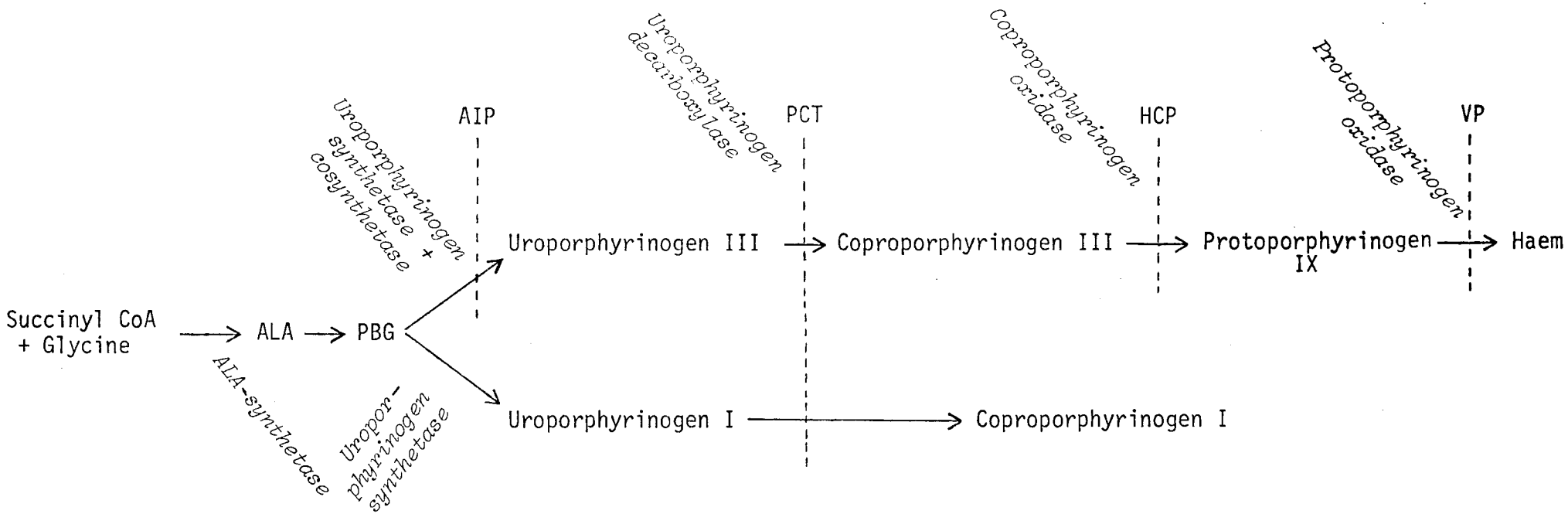
ERYTHROPOIETIC PORPHYRIAS

1) Congenital Porphyria

2) Erythropoietic Protoporphyria

* Acute attacks are characteristic of these types of porphyrias.

FIGURE 1.16. An outline of the haem biosynthetic pathway showing the different enzymatic defects associated with the human hepatic porphyrias.



AIP = Acute Intermittent Porphyria; HCP = Hereditary Coproporphyria; VP = Variegate Porphyria; PCT = Porphyria Cutanea Tarda.

----- indicates the position of the enzymatic defect in the different porphyrias.

Intermediates that are excreted in excess in hepatic porphyrias are those preceding the enzymatic block.

TABLE 1.5

ABNORMALITIES OF THE HAEM BIOSYNTHETIC PATHWAY ASSOCIATED WITH THE HUMAN HEPATIC PORPHYRIAS

PORPHYRIA	ENZYME DEFECT	HAEM PRECURSORS EXCRETED IN EXCESS
Acute Intermittent Porphyria	↓ Uroporphyrinogen synthetase (↑ ALA-synthetase)+	↓ δ-Aminolevulinic acid ↑ Porphobilinogen*
Variegate Porphyria	↓ Protoporphyrinogen oxidase (↑ ALA-synthetase)+	↑ δ-Aminolevulinic acid ↑ Porphobilinogen ↑ Uroporphyrin III ↑ Coproporphyrin III ↑ Protoporphyrin IX*
Hereditary Coproporphyria	↓ Coproporphyrinogen oxidase (↑ ALA-synthetase)+	↑ δ-Aminolevulinic acid ↑ Porphobilinogen ↑ Uroporphyrin III ↑ Coproporphyrin III*
Porphyria Cutanea Tarda	↓ Uroporphyrinogen Decarboxylase	↑ Uroporphyrin * ↑ 7-Carboxylic porphyrin ↑ 6-Carboxylic porphyrin ↑ 5-Carboxylic porphyrin

* The haem precursor immediately preceding the enzymatic block is excreted in the greatest amounts.

+ Where an increase in the activity of ALA-synthetase is found, it is generally thought to be a secondary effect, resulting from the decrease in haem levels due to the block in the haem biosynthetic pathway.

which lowers the rate of synthesis of haem could interfere with the feedback control of haem biosynthesis and lead to the stimulation of the activity of ALA-synthetase (227). Inhibition of haem synthesis at one of the steps in the biosynthetic chain will lead to the differences in the excretory products observed in the various types of porphyrias; the major intermediate which will accumulate is that immediately preceding the enzymatic block (Fig. 1.16) (Table 1.5) (228).

1.8. CHEMICALS AND PORPHYRIA

Studies of experimental hepatic porphyrias have provided valuable insight into the mechanisms by which the pathways of haem biosynthesis and biodegradation are regulated in the liver. The experimental porphyrias also serve as convenient model systems for studying the genetic hepatic porphyrias in man in as much as a number of the experimental porphyrias induced by drugs and chemicals resemble the different types of genetic porphyria (Table 1.6) (228).

It has been established that there are two main ways in which drugs and xenobiotics can be implicated in the derangement of liver porphyrin metabolism: (1) They can induce a condition of hepatic porphyria in normal human individuals or in experimental animals in the absence of a genetic disposition to porphyria, or (2) they can precipitate an acute attack of hepatic porphyria in patients who are carriers of a genetic defect for acute porphyria in the latent state (220). Examples of the ability of chemicals to derange haem biosynthesis in these two ways will be discussed below (Sections 1.8.1 - 1.8.2).

TABLE 1.6

EXPERIMENTALLY INDUCED PORPHYRIAS WHICH ARE USED AS MODEL SYSTEMS FOR STUDYING HUMAN PORPHYRIAS

AGENT USED TO INDUCE PORPHYRIA IN EXPERIMENTAL ANIMALS	THE HUMAN PORPHYRIA FOR WHICH THE EXPERIMENTAL PORPHYRIA IS USED AS A MODEL SYSTEM
DDC	Variegate Porphyria
Griseofulvin	Variegate Porphyria
Hexachlorobenzene	Porphyria Cutanea Tarda
Phenyl hydrazine	Congenital Erythropoietic Porphyria
U.V. light exposure	Congenital Erythropoietic Porphyria
AIA	Experimental Porphyria (does not resemble any specific human genetic porphyria)

No chemical model of porphyria exactly replicates any of the genetic porphyrias but merely provides an approximate biochemical simulation of these conditions.

1.8.1. Chemically induced porphyria

The first reported case of the ability of chemicals to induce a state of porphyria in experimental animals and man was in 1889, when Stokvis observed porphyric symptoms in patients with sulfonal poisoning (168, 229). However, Stokvis could not be certain that these patients did not have a genetic defect of haem biosynthesis in the latent state (190).

1.8.1.1. Hexachlorobenzene (HCB) (Fig. 1.17)

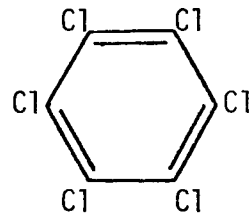
The outbreak of porphyria in Turkey in 1956 was the first direct evidence for the occurrence of a purely acquired and not genetically predetermined form of hepatic porphyria in man (190). This outbreak was caused by ingestion of hexachlorobenzene-treated wheat by several genetically distinct population groups (230-232). Subsequent experiments with laboratory animals have shown that hexachlorobenzene produces a form of hepatic porphyria which closely resembles Porphyria Cutanea Tarda (Tables 1.5, 1.6) (233) in that the activity of uroporphyrinogen decarboxylase is decreased (234).

1.8.1.2. Dicarbethoxydihydrocollidine (DDC) (Fig. 1.17)

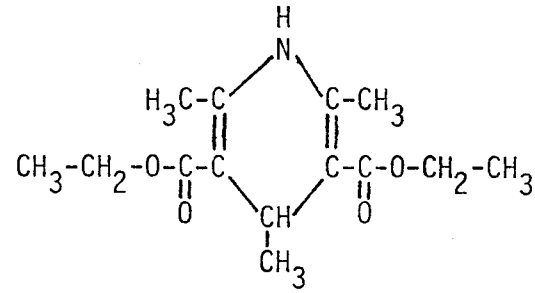
DDC was used in 1959 by Solomon and Figge (235) to produce experimental porphyria. It has been established that DDC will induce a porphyria in experimental animals which closely resembles human hepatic Variegate Porphyria (Tables 1.5, 1.6) (228) in that the activity of the enzyme ferrochelatase is decreased, resulting in an increase in the excretion of protoporphyrin IX (236) and an increase in the levels of ALA-synthetase (150).

FIGURE 1.17. Chemical inducers of hepatic porphyria.

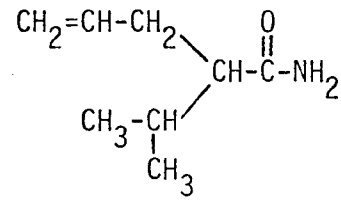
Hexachlorobenzene
(HCB)



Dicarbethoxydihydrocollidine
(DDC)



Allyl-iso-propylacetamide
(AIA)



Only very recently has the mechanism whereby DDC inhibits ferrochelatase been established. DDC treatment promotes the accumulation in the liver of a modified porphyrin with strong inhibitory activity towards ferrochelatase (237-239). The structure of this porphyrin has been determined to be N-methylprotoporphyrin IX (131, 240, 241) formed by covalent attachment of a methyl group from DDC to one of the pyrrole nitrogens of the porphyrin nucleus of liver haem (242). Contrary to earlier reports which suggested that no part of the DDC molecule was attached to the modified porphyrin (238), the methyl group of N-methylprotoporphyrin IX has now been shown conclusively to originate from the 4-methyl substituent of DDC (Fig. 1.17) (131, 240). All four isomers of N-methylprotoporphyrin IX are equally potent inhibitors of ferrochelatase (241); however, the nitrogen in pyrrole ring A of protoporphyrin IX appears to be preferentially alkylated by DDC *in vivo* (243).

Since DDC does not inhibit ferrochelatase *in vitro* (236) and activation of DDC by hepatic microsomal cytochrome P-450 is required for the formation of the modified porphyrin (242), it has been postulated that the 4-methyl substituent of DDC is eliminated during the cytochrome P-450 catalyzed oxidation of DDC (131). However, the exact mechanism of formation of N-methylprotoporphyrin IX is at present unknown.

The manner in which the modified porphyrin inhibits ferrochelatase is thought to involve the binding of N-methylprotoporphyrin IX to the porphyrin binding site at the active site of the enzyme (238), thus interfering with the normal incorporation of Fe^{2+} into protoporphyrin and accounting for the accumulation of protoporphyrin IX and the induction of porphyria in DDC treated animals (238). Since N-methyl-

protoporphyrin IX is also present in the livers of untreated animals (240), it has been proposed that the endogenous presence of N-methyl-protoporphyrin IX may be important in the regulation of haem biosynthesis (240). The source of the methyl group for endogenous N-methylprotoporphyrin IX is unknown at this time, but it may be possible that it is derived from endogenous metabolites similar in structure to DDC. On the other hand, there may be xenobiotics present in food or air which contribute to the production of endogenous N-methyl-protoporphyrin IX (240).

1.8.1.3. Allyl-iso-propylacetamide (AIA) (Fig. 1.17)

AIA was first used by Goldberg and Rimington in 1955.

AIA is a chemical which is commonly used to induce experimental porphyria in animals. A single injection of AIA causes a striking increase in hepatic ALA-synthetase activity (215, 220) and a marked increase in the excretion of haem precursors in the urine and faeces of these animals (244). AIA is also known to cause a transient decrease in the levels of hepatic microsomal cytochrome P-450 which precedes the increase in ALA-synthetase activity (245, 246). The decrease in the levels of cytochrome P-450 is due to the interaction of AIA with the haem moiety of cytochrome P-450.

AIA degrades the haem moiety of hepatic microsomal cytochrome P-450 in a highly specific, suicidal process (121) which requires catalytically competent enzyme, NADPH and oxygen (127). The specificity of this process is attested to by the fact that AIA selectively destroys only the haem moiety of cytochrome P-450 whereas the apoprotein moiety appears to be unchanged (125, 126). Since AIA is primarily metabolized by and exclusively degrades the major phenobarbital-inducible form of

cytochrome P-450 (127, 132), it appears that a reactive metabolite of AIA, generated in situ, selectively destroys the haem moiety of this isoenzyme (121) before diffusing out of the active site (136).

The product of the AIA mediated degradation of cytochrome P-450 is an abnormal green pigment which accumulates in the liver of AIA treated animals and is not catabolized by haem oxygenase (248). Although the exact structure of the pigment has not been determined, it it thought to be an N-substituted porphyrin (137) which is formed by 1:1 covalent attachment of a portion of the AIA molecule to protoporphyrin IX (249), the protoporphyrin IX being derived from the prosthetic haem moiety of inactivated cytochrome P-450 (249). This abnormal porphyrin differs from the N-substituted porphyrin formed by DDC treatment (Section 1.8.1.2) in the nature of the substituent on protoporphyrin IX (127, 131, 238) and by the fact that it does not inhibit ferrochelatase (238).

Since the AIA mediated degradation of cytochrome P-450 and the formation of the abnormal porphyrin result in the loss of haem from cytochrome P-450, the intact apoprotein which remains will draw haem from the central hepatic haem pool for the reconstitution of the holoenzyme (250). Thus, after AIA treatment, the apoprotein of cytochrome P-450 can be visualized as a catalytic centre for the degradation of newly synthesized endogenous haem (250) and in this way causes depletion of the hepatic regulatory haem pool, thereby decreasing the negative feedback control by haem on ALA-synthetase and resulting in the stimulation of ALA-synthetase activity and the development of hepatic porphyria (220).

1.8.2. Chemical precipitation of an acute attack

Many lipid-soluble drugs (Table 1.7) (255) can precipitate an acute attack of hepatic porphyria in patients who are carriers of a genetic defect in the latent state (220). Both *in vivo* and *in vitro*, barbiturates and many lipid-soluble drugs, alone, have relatively small inducing capacities for ALA-synthetase (228). These drugs are much more effective in stimulating hepatic ALA-synthetase if a partial block in liver haem biosynthesis has been established, such as in patients with a genetically determined defect of haem biosynthesis (Fig. 1.16) (Table 1.5) or in experimental animals treated with DDC (251). These results suggest that the sensitivity of patients with genetic porphyria of an acute variety to barbiturates and other lipid-soluble drugs may depend on the partial block in haem biosynthesis which already exists in their livers as a result of the genetic defect (252). This interpretation is supported by the finding that in tissue culture, inhibition of haem biosynthesis by several agents, will potentiate synergistically the induction of ALA-synthetase by drugs (253, 254).

1.9. THE EFFECTS OF CARBON DISULPHIDE ON HEPATIC HAEM METABOLISM AND HEPATIC MICROSOMAL CYTOCHROME P-450

In contrast to chemicals such as AIA and DDC which cause an increase in hepatic haem biosynthesis, carbon disulphide causes an increase in hepatic haem biodegradation (256) and a slight decrease in hepatic haem biosynthesis (256). However, the effects of carbon disulphide on hepatic haem metabolism are thought to be a consequence of the carbon disulphide mediated destruction of hepatic microsomal cytochrome P-450 (256).

TABLE 1.7

DRUGS UNSAFE FOR PORPHYRIC PATIENTS (255)

Dangerous drugs	Grading*	Contentious drugs+
Antimicrobial agents		
Dapsone	A	Chloramphenicol
Griseofulvin	A	Chloroquine
Pyrazinamide	A	Nitrofurantoin
Sulphonamides	A	Pyrimethamine
Colistin	B	Tetracyclines
Erythromycin	B	Rifampicin
Isoniazid	B	
Nalidixic acid	B	
Novobiocin	B	
Metronidazole	C	
Anaesthetic agents		
Barbiturates	A	Halothane
Chloroform	B	Ketamine
Fluroxine	B	Propanidid
Alfathesin (alphaxalone)	B	Fentanyl
Methoxyflurane	B	
Etomidate	C	
Enflurane	C	
Lignocaine	C	
Anticonvulsants		
Barbiturates	A	
Hydantoins (phenytoin, mesantoin, ethotoin)	A	Clonazepam
Carbamazepine	A	Diazepam
Succinimides	A	Paraldehyde
Oxazolidinediones (tridione, paradione)	B	Sodium valproate
Sulthiame	B	
Minor tranquillizers/sedatives/hypnotics		
Glutethimide	A	Oxazepam
Methyprylone	A	Chlordiazepoxide
Meprobamate	A	Clonazepam
Isopropyl meprobamate	A	Diazepam
Flunitrazepam	B	
Nitrazepam	B	
Carbromal	B	
Ethchlorvynol	B	
Apronalide	B	
Ethinamate	C	
Major tranquillizers/anti-emetics		
Metoclopramide	B	

TABLE 1.7. (contd.)

Dangerous drugs	Grading*	Contentious drugs+
Psycho-analeptics		
Tranylcypromine	C	Imipramine
Pargyline	C	Amitriptyline
Antihistamines		
Dimenhydrinate	B	
Cimetidine	C	
Hormone preparations		
Metyrapone	D	Corticosteroids Oestrogens Androgens Progestogens
Antidiabetic agents		
Sulphonylureas	A	
Cardiovascular/respiratory preparations		
Theophylline	B	
Diuretics		
Furosemide	B	Thiazides
Spironolactone	C	
Antihypertensive agents		
Alpha-methyldopa	A	Hydrallazine
Clonidine	D	
Phenoxybenzamine	D	
Anticoagulants		
Analgesics/antirheumatics		
Narcotics		
Pentazocine	A	Pethidine
Non-steroidal anti-inflammatory agents		
Dipyrone	A	
Isopropyl antipyrine	A	
Amidopyrine	A	
Antipyrine	A	
Dichloralphenazone	A	
Phenylbutazone	A	
Flufenamic acid	B	
Ketoprofen	B	
Diclophenac sodium	B	
Azapropazone	C	
Anti-gout agents		Probenecid

TABLE 1.7. (contd.)

Dangerous drugs	Grading*	Contentious drugs+
Specific antirheumatic agents		
Gold	C	
Muscle relaxants/antispasmodics		
Dipyrone/avapyrazone	A	
Hyoscine butyl bromide dipyrone	A	
Sodium phenyl dimethyl pyrazolone	A	
Metoclopramide	B	
Pancuronium	B	
Anticholinesterases		
Antineoplastic agents		
Busulphan	C	
Cyclophosphamide	C	
Chlorambucil	C	
Miscellaneous		
Diethylpropion	B	Ethanol
Eucalyptol	B	

* A - based on human experience reported by 3 or more authors.
B - based on human experience reported by 1 or 2 authors.
C - based on experiments in animal models.
D - based on experiments on chick embryo liver cell culture or in ovo.

+ Contentious drugs are those drugs for which conflicting data are available (255).

The metabolism of carbon disulphide by cytochrome P-450 is thought to produce one or more reactive species of sulphur which can bind covalently to the apoprotein of cytochrome P-450 (256, 257). Consequently, the affinity of the modified apoprotein for haem is reduced and carbon disulphide-modified cytochrome P-450 dissociates into haem plus modified apoprotein moieties (120, 256). The appearance of cytochrome P-420 in the microsomes from carbon disulphide treated animals (258, 259) supports this theory since some of the haem which is lost from cytochrome P-450 presumably becomes bound to microsomal lipid, giving rise to the spectrum of cytochrome P-420 (Fig. 1.10) (134).

Thus, the carbon disulphide mediated dissociation of cytochrome P-450 results in an increase of free haem in the liver and in the regulatory haem pool in the cytosol (a migration of radioactive haem from the microsomes to the cytosol has been observed after carbon disulphide treatment (256)). The increased levels of haem in the regulatory haem pool cause an increase in the activity of hepatic haem oxygenase (256) and a slight decrease in the activity of hepatic ALA-synthetase (256). These effects are in contrast to the effects observed after AIA treatment where a decrease in hepatic haem levels results in an increase in hepatic ALA-synthetase (Section 1.8.1.3) (220, 250).

1.10. PURPOSE OF PROPOSED INVESTIGATIONS

As shown in this Introduction, there are several ways in which chemicals can interfere with hepatic haem metabolism. The primary effects of these chemicals on haem metabolism appear to be classifiable as follows:

(A) The compound can mediate the modification of the haem moiety of hepatic microsomal cytochrome P-450, resulting in a decrease in the

levels of haem in the regulatory haem pool and the subsequent stimulation of hepatic haem biosynthesis (cf. AIA (Section 1.8.1.3)).

(B) The chemical may stimulate the dissociation of hepatic microsomal cytochrome P-450 into haem plus modified apoprotein, resulting in an increase of haem in the regulatory haem pool and thereby increase hepatic haem biodegradation (cf. carbon disulphide (Section 1.9)).

(C) Finally, a compound may inhibit one of the key enzymes of haem biosynthesis, resulting in a block in the haem biosynthetic pathway and a decrease in the negative feedback control of haem on ALA-synthetase (cf. DDC (Section 1.8.1.2)).

In this thesis, we have attempted to gain some further insight into the mechanism of control of haem metabolism and the production of experimental porphyria by examining the role of hepatic microsomal cytochrome P-450 in controlling the pathways for the biosynthesis and biodegradation of haem. More specifically, the roles of the apoprotein and haem moieties of this enzyme in controlling haem metabolism were investigated as follows:

Firstly, two compounds which decrease the haem of cytochrome P-450, e.g. fluroxene and trichloroethylene, were utilized to determine the effects of the haem of this enzyme on the regulation of hepatic haem biosynthesis; and

Secondly, one compound which apparently affects both the haem and apoprotein moieties of cytochrome P-450, viz. halothane, was utilized to ascertain the play-off of these two effects on hepatic haem biosynthesis and haem biodegradation.

II MATERIALS

Fluroxene (2,2,2-trifluoroethyl vinyl ether) was obtained from Ohio Medical Products, Madison, Wisconsin, U.S.A. Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) was from I.C.I. Ltd., Johannesburg, South Africa. Trichloroethylene and tetrachloroethylene were purchased from Merck Chemicals, Darmstadt, Germany. The inducing agents sodium phenobarbital and 3-methylcholanthrene were supplied by Maybaker Ltd., Port Elizabeth, South Africa and Eastman Kodak Company, Rochester, New York, U.S.A., respectively. NADPH, crystalline bovine serum albumin and the components of the NADPH-generating system (NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) were purchased from Miles Laboratories, Cape Town, South Africa. Haemin (haematin HCl) and BF_3/methanol were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. δ-Aminolevulinic acid hydrochloride (ALA-HCl) and porphobilinogen were supplied by Sigma Chemical Company, Saint Louis, Missouri, U.S.A. Cyclohexanone and acetylacetone were obtained from Merck, Darmstadt, Germany. Afrox Limited, Cape Town, South Africa, supplied cylinders of pure gases. 2,2,2-Trifluoroethyl ethyl ether (TFEE) was prepared by hydrogenation of fluroxene as described by Ivanetich et al. (135). [1,4-^{14}C]-Succinic acid (50.7 mCi/mmole) was purchased from New England Nuclear, Boston, Massachusetts, U.S.A. Dicarbethoxydihydrocollidine (DDC) and resofurin were obtained from Eastman Kodak Company, Rochester, New York, U.S.A. Ethoxyresofurin was prepared from resofurin as described by Burke and Mayer (260). Metyrapone [2-methyl-1,2-bis-(3-pyridyl)-1-propane] was a gift from Ciba-Geigy Ltd., Basle, Switzerland. Sephadex LH-20 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals used were of the highest purity commercially available. Water was glass distilled and deionized.

III METHODS

3.1. EXPERIMENTAL ANIMALS

Male Wistar rats weighing between 190 g and 210 g were used in all experiments, except where indicated. In the latter case, male Long Evans rats weighing between 190 g and 210 g were used. The animals were allowed free access to Epol Laboratory Chow (protein minimum 20%, fat 2.5%, fibre maximum 6%, calcium 1.4%, phosphorus 0.7%) unless otherwise indicated and were allowed free access to water at all times.

3.2. TREATMENT OF ANIMALS IN VIVO

Cytochrome P-450 was induced by intraperitoneal injections of sodium phenobarbital (80 mg/kg/day in 0.9% saline for one day) or 3-methylcholanthrene (40 mg/kg/day in corn oil for three consecutive days). Animals which had been induced with sodium phenobarbital were administered fluroxene, halothane or trichloroethylene at a dose of 1 ml/kg. Animals pretreated with 3-methylcholanthrene received 4 ml/kg of fluroxene. Uninduced animals received either 4 ml/kg or 6 ml/kg of fluroxene. However, halothane or trichloroethylene was administered to uninduced animals at a dose of 2 ml/kg*. TFEE or tetrachloroethylene was injected into phenobarbital pretreated rats at a dose of 1 ml/kg. To both the 3-methylcholanthrene and phenobarbital pretreated animals, the compounds were administered 24 hours after the last injection of inducing agent. In the experiments where the anaesthetic agent was administered repeatedly, a dose of 1 ml/kg was administered to uninduced rats at 48 hour intervals. In all cases the compound was administered

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* These doses of the anaesthetic agents represent the maximum doses that could be administered to the Wistar rats without fatality.

neat by intraperitoneal injection.

In one experiment, ALA-synthetase was induced by a single intraperitoneal injection of DDC (100 mg/kg in Arachis oil). Fluroxene was administered to these rats at a dose of 4 ml/kg immediately after the DDC treatment.

When the effects of halothane or fluroxene as a function of time were studied in vivo, animals were starved 24 hours prior to fluroxene or halothane treatment and sacrificed at 0, 1, 5 and 10 hours after halothane or fluroxene treatment or at equivalent times in controls. In all other cases, animals were starved 24 hours before being sacrificed by cervical fracture and, where required, the livers were removed immediately and used promptly.

3.3. IN VITRO STUDIES

For in vitro experiments rats were pretreated with sodium phenobarbital (80 mg/kg in 0.9% NaCl per day) for three consecutive days and fasted overnight prior to being sacrificed. Immediately upon sacrifice of the animals microsomes were prepared as described below (Section 3.4). Halothane (18 mM) or fluroxene (6 mM) was dispersed in microsomal suspensions (2 mg protein/ml) by vortex mixing for 30 sec. NADPH-generating system (65) and EDTA (0.2 mM) were added, and reaction mixtures were incubated at 30°C with shaking at 60 cycles/min.

3.4. PREPARATION OF MICROSOMES

Immediately after the animals were sacrificed, microsomes were prepared from fresh rat liver homogenates at 4°C by differential ultracentrifugation

according to the method of Holtzman and Carr (261). The microsomes were finally suspended at a concentration of 2 mg microsomal protein/ml in 0.02 M Tris-HCl, pH 7.4, and were used immediately after preparation (16). The protein concentration of the microsomal suspension, liver homogenate and post-mitochondrial supernatant was determined by the method of Lowry et al. (262) as modified by Chaykin (263), using bovine serum albumin as a standard.

3.5. LIVER ASSAYS

3.5.1. Microsomal assays

3.5.1.1. Cytochrome P-450.

The concentration of cytochrome P-450 in hepatic microsomes was determined from measurements of the difference spectra of carbon monoxide-ferrocytochrome P-450 versus ferrocytochrome P-450, as described by Omura and Sato (264). An extinction coefficient of $\varepsilon = 91\ mM^{-1}$ was used for $A_{450\ nm} - A_{490\ nm}$.

3.5.1.2. Haem.

The concentration of microsomal haem was determined by the reduced pyridine haemochromogen as described by Omura and Sato (264). An extinction coefficient of $\varepsilon = 34.7\ mM^{-1}$ was used for $A_{557\ nm} - A_{575\ nm}$.

3.5.1.3. Metyrapone-ferrocytochrome P-450 complex.

The concentration of the major phenobarbital-inducible form of cytochrome P-450 was determined in microsomes from phenobarbital treated rats from measurements of the difference spectra of metyrapone-ferrocytochrome P-450 versus ferrocytochrome P-450 as described by Luu-The et al. (265), but cf. (282). An extinction coefficient of $\varepsilon = 52\ mM^{-1}$ was used for $A_{446\ nm} - A_{490\ nm}$.

3.5.1.4. Ethoxyresofurin deethylase activity.

The concentration of the major 3-methylcholanthrene-inducible form of cytochrome P-450 was determined from the rate of O-deethylation of ethoxyresofurin to resofurin, and monitored by recording the increase in fluorescence associated with the formation of resofurin as described by Burke and Mayer (260). $\lambda_{excitation}$ = 510 nm; $\lambda_{emission}$ = 586 nm

3.5.1.5. Haem oxygenase.

The method described by Tenhunen et al. (204) was used with minor modifications to determine the activity of hepatic haem oxygenase. The sample cuvette contained 18 000 g supernatant (approximately 5 mg protein), haemin* (17 µM), NADPH (0.6 mM), and potassium phosphate buffer, pH 7.4 (90 mM) in a total volume of 3 ml. In the reference cuvette NADPH was replaced by an equivalent volume of 0.1 M potassium phosphate buffer, pH 7.4. Enzyme activity was determined from the increase in absorbance at 468 nm over 10 minutes at 37°C. The enzyme activity was calculated from the rate of formation of bilirubin as follows:

$$\text{Bilirubin formed (nmoles/mg microsomal protein / 10 min)} = \frac{\Delta A_{468\ nm} / 10\ min}{\varepsilon_{468\ nm}} \times \frac{V^t}{V^\circ} \times \frac{1}{\text{mg microsomal protein/ml}}$$

$\Delta A_{468\ nm}$ / 10 min = the change in absorbance at 468 nm over 10 minutes

$\varepsilon_{468\ nm}$ = 30 mM^{-1} (204)

V^t = total volume in the reaction cuvette (3 ml)

V° = volume of supernatant used (ml)

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* A stock solution of haemin (1.0 mM) was prepared as follows: 10 mg haemin was dissolved in 5 ml 0.1 N NaOH containing 12 mg Tris and 222 mg NaCl. To this solution was added 10 ml of 3% (w/v) bovine serum albumin, and the pH was adjusted to 7.4.

3.5.2. Uroporphyrinogen synthetase

The activity of uroporphyrinogen synthetase in post-mitochondrial supernatant was determined from the loss of porphobilinogen as described by Hutton and Gross (266). For spectrophotometric readings, the reaction mixtures obtained by the above method were diluted 1/15 (v/v) with distilled H_2O. A volume of the diluted samples was mixed with an equal volume of modified Ehrlich's reagent (Section 3.7.1), and the absorbance at 553 nm was determined after 30 minutes in a Gilford single beam spectrophotometer.

Enzyme activity was calculated from the amount of porphobilinogen which remained in the sample after 60 minutes reaction time, compared to the amount of porphobilinogen in the sample at zero time.

$$\text{Porphobilinogen remaining } (\mu\text{moles}) = 0.31\ \mu\text{moles} - \frac{0.31\ \mu\text{moles}}{A^{o}_{553\ nm}} \times A^{60}_{553\ nm}$$

0.31 μmoles = total μmoles of porphobilinogen added to the sample at zero time

$A^{o}_{553\ nm}$ = absorbance at 553 nm of sample at zero time

$A^{60}_{553\ nm}$ = absorbance at 553 nm of sample after 60 minutes

3.5.3.1. ALA-synthetase.

Rat liver homogenized in 3 volumes of 0.9% NaCl containing 0.5 mM EDTA and 10 mM Tris-HCl (pH 7.4) was used for the determination of ALA-synthetase activity as described by Marver et al. (267,268). 0.5 ml of liver homogenate was incubated at 37°C for 30 minutes with 0.05 ml of 0.08 mM ATP and 1.5 ml of a solution containing 200 μM glycine, 20 μM EDTA and 150 μM Tris-HCl (pH 7.2). On termination of the reaction with 25% TCA, the ALA in the supernatant was converted to a pyrrole adduct by

condensation with acetylacetone. The pyrrole adduct was applied to a Dowex-1-acetate column (1x7 cm) equilibrated with 0.05 M sodium acetate pH 4.6. The column was washed with 10 ml of n-butanol containing 0.01 M ammonium hydroxide and 10 ml of 1 M acetic acid. The ALA pyrrole was eluted with 10 ml of a mixture of glacial acetic acid and methanol (1:2).

The eluate (ca. 8 ml) was reacted with an equal volume of modified Ehrlich's reagent (Section 3.7.1) for 30 minutes, at which time the absorbance at 556 nm was measured in a Gilford single beam spectrophotometer. One ml of a standard solution of ALA (8 µg/ml) was used (Section 3.7.1) to correct for losses sustained during the purification procedure.

ALA-synthetase activity was calculated from the amount of ALA formed during the incubation as follows:

$$\text{Enzyme activity } (\mu g \text{ ALA formed/mg protein/30 min}) = \frac{A^{\text{unknown}}_{556\text{ nm}}}{A^{\text{std}}_{556\text{ nm}}} \times \frac{8}{3} \times \frac{V^{E}_{\text{unknown}}}{V^{E}_{\text{std}}} \times \frac{1}{\text{mg microsomal protein/ml}}$$

$A^{\text{unknown}}_{556\text{ nm}}$ = Absorbance at 556 nm of unknown

$A^{\text{std}}_{556\text{ nm}}$ = Absorbance at 556 nm of standard

V^{E}_{unknown} = Volume of eluant of unknown

V^{E}_{std} = Volume of eluant of standard

3.5.3.2. Assay for ALA-synthetase using radioisotopes.

This assay was performed using radioactively labelled [1,4-^{14}C]-succinic acid according to the method described by Blekkenhorst (269). A known amount of [1,4-^{14}C]-succinic acid was incubated with rat liver homogenate and the amount of [^{14}C]-ALA formed was used as a measure of the activity

of ALA-synthetase. This method was only used for the determination of ALA-synthetase activity in DDC treated rats.

3.6. COLLECTION OF URINE AND FAECES

Immediately after treatment with the compound or at an equivalent time in controls, animals were housed in groups of three in perspex metabolic cages for collection of urine and faeces. Animals were starved but allowed water ad libitum over the 24 hour collection period, after which time the animals were sacrificed immediately. In the experiments where the anaesthetic was administered repeatedly over a period of two to three weeks, the animals were allowed free access to food until 24 hours before sacrifice and had free access to water throughout the experiment. For these experiments, the urine and faeces were collected over periods of 48 hours. Urine and faeces were stored frozen for a maximum period of 7 days.

3.7. QUANTIFICATION OF HAEM PRECURSORS

3.7.1. Estimation of urinary ALA and porphobilinogen

The colorimetric method described by Mauzerall and Granick (193) was used for the quantitative determination of urinary ALA and porphobilinogen. This method makes use of the retention of porphobilinogen and ALA on anionic and cationic ion exchange resins, respectively. Once eluted from their respective columns, ALA, in the pyrrole form, and porphobilinogen are determined colorimetrically with modified Ehrlich's reagent (1 g p-dimethylaminobenzaldehyde and 8 ml 70% perchloric acid in 50 ml glacial acetic acid). The modified Ehrlich's reagent was allowed to react with porphobilinogen and ALA (in the

pyrrole form) for 15 minutes, after which time, the absorbance at 555 nm and at 556 nm for porphobilinogen and ALA respectively, was measured immediately on a Gilford single beam spectrophotometer.

In order to estimate the percentage recovery of ALA, 1 ml of a standard solution containing 8 µg of ALA was treated exactly as were the urinary samples and was subsequently analysed for ALA content. Reported values were thereby corrected for losses of ALA sustained during the purification procedure.

The equation given by Mauzerall and Granick (193) was used to determine urinary porphobilinogen levels:

$$\text{Urinary porphobilinogen } (\mu g) = A_{555\ nm} \times \frac{0.328 \times V^t \times M_r}{V^\circ}$$

V^t = total volume of urine collected (ml)

V° = amount of urine placed on column (ml)

$A_{555\ nm}$ = absorbance at 555 nm

M_r = molecular weight of porphobilinogen.

The following equation was used to determine urinary ALA content:

$$\text{Urinary ALA } (\mu g) = \frac{A_{556\ nm}\ \text{unknown}}{A_{556\ nm}\ \text{standard}} \times 8\ \mu g/ml \times V^t$$

V^t = total volume of urine collected (ml)

$A_{556\ nm}$ unknown = absorbance of unknown sample at 556 nm

$A_{556\ nm}$ standard = absorbance of standard sample at 556 nm.

3.7.2. Determination of urinary coproporphyrin and uroporphyrin

3.7.2.1. Coproporphyrin.

The method of Schwartz et al. (270) as modified by Holti et al. (271) was used with several minor modifications: 5 ml of urine was shaken with 0.5 ml of glacial acetic acid and 20 ml of diethyl ether. On separation of the two phases, coproporphyrin is found in the ether layer whereas uroporphyrin is retained in the aqueous layer. The aqueous layer was extracted with ether three or four times, and the ether extracts were combined for the determination of coproporphyrin. The combined ether extracts were washed once each with 20 ml of 0.5% sodium acetate, 20 ml of 0.005% iodine solution* and 10 ml of H_2O. The acetate, iodide and aqueous washes were combined with the initial glacial acetic acid-water layer and were set aside for the determination of uroporphyrin.

The coproporphyrin was extracted repeatedly from the washed ether layer using 2 ml aliquots of 1.5 M HCl, until no further fluorescence was observed in the extracts. The total volume of acid was recorded, and the solution was filtered when necessary.

The coproporphyrin content was calculated from the absorbances at 380 nm, 430 nm and at the Soret peak (ca. 405 nm).

The correction formula of Rimington and Sveinsson (272) as modified by With (273), with an extinction coefficient given by Furhop and Smith (274) was used to determine urinary coproporphyrin levels.

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* Iodine solution is used to oxidise coproporphyrinogen to coproporphyrin.

$$\text{Coproporphyrin } (\mu g) = \left[2A_{max} - (A_{430\ nm} + A_{380\ nm})\right] \times 0.728 \times \frac{V^1}{V^o} \times V^t$$

V^1 = volume of HCl (ml)

V^o = volume of urine used (ml)

V^t = total volume of urine (ml)

A_{max} = absorbance at Soret maximum

$A_{430\ nm}$ = absorbance at 430 nm

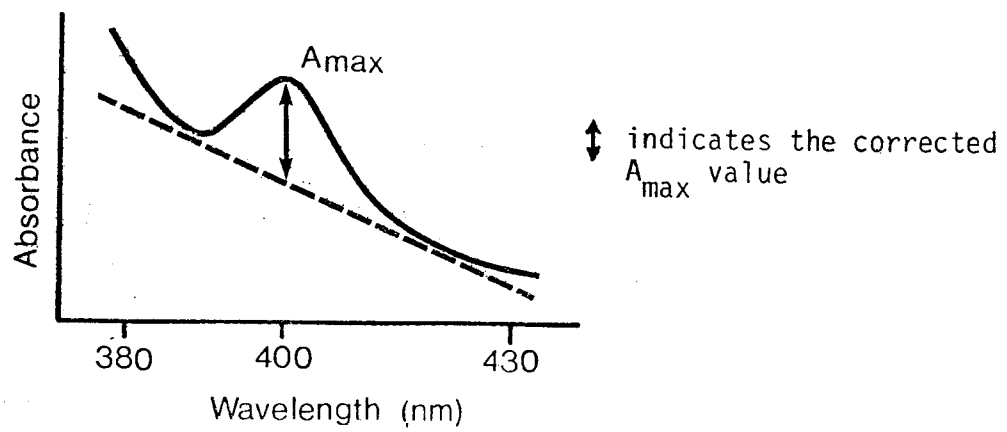
$A_{380\ nm}$ = absorbance at 380 nm

3.7.2.2. Uroporphyrin.

The method described by Dresel et al. (275) was used for the determination of urinary uroporphyrin. The combined aqueous washes from the coproporphyrin determination were extracted with cyclohexanone. To the cylcohexanone extracts, diethyl ether was added, and the uroporphyrin was extracted finally into 2% aqueous HCl. The uroporphyrin content of the HCl extracts was calculated from the absorbance of the Soret peak.

The correction formula described by Rimington and Sveinsson (272) was used for determination of urinary uroporphyrin levels, with a slight modification to take into account the impurities present in the solutions. A tangent to the background slope was drawn, and the absorbance between the curve and the tangent at the Soret peak was subtracted from the total absorbance at the Soret peak (A_{max}) shown in Figure 3.1.

Figure 3.1. Diagram of method used for calculation of urinary uroporphyrin



At high concentrations of urinary uroporphyrin (ca. 6 µg) our results were consistent with results obtained using the Rimington and Sveinsson formula (272). However, at low concentrations of urinary uroporphyrin, below ca. 0.5 µg, our method of calculation proved to be more reproducible.

$$\text{Uroporphyrin } (\mu g) = 2[A_{max}] \times 0.855 \times \frac{V^2}{V^o} \times V^t$$

V^2 = volume of 2% HCl (ml)

V^o = volume of urine used (ml)

V^t = total volume of urine collected (ml)

A_{max} = absorbance at Soret maximum (ca. 405 nm)

3.7.3. Determination of coproporphyrin and protoporphyrin in faeces

The method described by Holti et al. (271) was used for the determination of faecal coproporphyrin and protoporphyrin following minor modifications: The combined ether extracts of the faeces were washed once each with 20 ml saturated sodium acetate, 20 ml 0.005% iodine solution and 20 ml H_2O. The coproporphyrin was extracted from the ether phase into 0.1 M HCl, and the protoporphyrin was subsequently repeatedly extracted using 1.5 M HCl. The absorbance spectra of the coproporphyrin and protoporphyrin extracts were recorded over the range of 350 nm to 450 nm.

Stool porphyrin content was reported per dry weight of faeces in all cases. The correction formulas described by Rimington and Sveinsson (272) as modified by With (273) were used for calculation of both the coproporphyrin and protoporphyrin content of the faeces.

Coproporphyrin (µg/g dry weight of faeces)

$$= [2A_{max} - (A_{430\ nm} + A_{380\ nm})] \times 0.730 \times \frac{V^1}{\text{wet weight of faeces used}} \times \frac{\text{wet weight of faeces}}{\text{dry weight of faeces}}$$

V^1 = volume of 0.1 M HCl (ml)

A_{max} = absorbance at Soret maximum (ca. 405 nm)

$A_{430\ nm}$ = absorbance at 430 nm

$A_{380\ nm}$ = absorbance at 380 nm

Protoporphyrin (µg/g dry weight of faeces)

$$= [2A_{max} - (A_{430\ nm} + A_{380\ nm})] \times 1.080 \times \frac{V^2}{\text{wet weight of faeces used}} \times \frac{\text{wet weight of faeces}}{\text{dry weight of faeces}}$$

V^2 = volume of 1.5 M HCl (ml)

A_{max} = absorbance at Soret maximum (ca. 408 nm)

$A_{430\ nm}$ = absorbance at 430 nm

$A_{380\ nm}$ = absorbance at 380 nm

3.8. PREPARATION AND ISOLATION OF THE GREEN PIGMENT

Administration of fluroxene to rat liver *in vivo* or *in vitro* results in the formation of a green pigment thought to be formed by attachment of fluroxene (or part thereof) to the prosthetic haem of cytochrome P-450 (132,276).

3.8.1. Preparation *in vitro* and isolation

The green pigment was prepared *in vitro* by incubation of fluroxene (6 mM) with hepatic microsomes from phenobarbital induced Wistar or Long Evans rats, EDTA and NADPH-generating system for 30 min, as described in

Section 3.3. At the end of the reaction period, excess fluroxene was removed by bubbling with nitrogen for 15 min at room temperature.

The green pigment prepared as described above was isolated by a combination of the methods described by McDonagh et al. (277) and Ortiz de Montellano et al. (278). The green pigment was extracted from hepatic microsomes (approximately 500 ml) with 3 volumes of ethyl acetate:acetic acid (4:1) (v/v). The extract was filtered, washed three times with aqueous saline (7.5% (w/v)) and evaporated to near dryness (ca. 5 ml). A small volume of chloroform (1-3 ml) was added to the remaining brown oil. The chloroform solution was methylated with 10 ml of BF_3/methanol (14% (w/v)) and the mixture was refluxed under nitrogen for 15 min. Upon cooling the reaction mixture, the methylated product was extracted into chloroform (80 ml) and washed twice with H_2O (50 ml) and once with 1% (w/v) sodium bicarbonate (50 ml). After drying over sodium sulphate, the chloroform layer was reduced to 1-2 ml by rotary evaporation. Purification of the methylated green pigment was performed using the following procedures:-

(1) HPLC - using a Whatman Partisil 10 PAC column eluted with tetrahydrofuran : hexane : methanol (5:5:1 by volume) and then with methanol : tetrahydrofuran (4:1 (v/v)); and/or

(2) TLC - using silica gel plates (20 cm x 20 cm : 0.2 mm thickness), developed in chloroform : kerosene : methanol (20:5:3 by volume). The developed plates were dried under a stream of cold air, the spots were located by their colour under a uv light, and the methylated product was extracted off the silica gel into chloroform: methanol (1:1 (v/v)).

The zinc complex of the methylated green pigment was prepared as described by Doss (279) and chromatographed using silica gel. The plates (20 cm x 20 cm : 0.2 mm thickness) were developed in 20% acetone/chloroform (v/v). The developed plates were treated as described above and the zinc complex was extracted off the silica gel into chloroform : methanol (1:1 (v/v)).

3.8.2. Preparation in vivo and isolation

The green pigment was prepared in vivo using phenobarbital induced Wistar rats injected with fluroxene (2 ml/kg) as described in Section 3.2 and killed 1 hour later. The method described by De Matteis et al. (238) was used to isolate the green pigment.

The livers of 4 treated rats were pooled and homogenised in two volumes of 0.25 M sucrose. The homogenate (approx. 150 ml) was washed twice with an equal volume of acetone and centrifuged at 2000 rpm for 10 min. The pellet was extracted twice with 100 ml of acetone : HCl (120 : 0.2 (v/v)) and centrifuged as described above. The extracts were combined, and the acetone was removed by rotary evaporation at 30°C. Two to three ml of H_2O were added to the HCl extract. This caused precipitation of haem and protoporphyrin, which were removed by centrifugation at 2000 rpm for 10 min. The green supernatant was applied to a Sephadex LH-20 column (20 x 1 cm) equilibrated with acetone : H_2O (1:1 (v/v)). The green pigment was eluted with 10 ml of acetone : H_2O (1:1 (v/v)).

All isolation steps were performed in the dark at room temperature.

3.9. SPECTROPHOTOMETRY

Unless otherwise stated, a Beckman 5230 spectrophotometer or a Unicam SP 1800 spectrophotometer coupled to a Unicam AR 25 recorder was used for all spectral measurements. The compartment adjacent to the photonmultiplier was used whenever samples were particulate.

3.10. CALCULATIONS AND STATISTICAL ANALYSIS

Results were given as means ± standard deviations and as percentages relative to control values. Where values were given in parentheses these represent results obtained on individual days. Determinations on each day were on the pooled livers, urine or faeces of three or more treated rats and three or more control rats. Assays were performed in duplicate to quadruplicate except for the determination of the urinary and faecal porphyrins, where sample size permitted single determinations only. In general, each result presented is an average of the determinations obtained on two or more days, the exception being for measurements of the levels of haem precursors in chronically treated animals where determinations were from a single day only.

Statistical analysis of the data was usually performed using the Student's t-test for unpaired data. However, where day to day variation in results was large in comparison to the difference between rats treated with the anaesthetic and control rats on a given day, the Student's t-test for paired data was used to assess the statistical significance. Where the paired test was used for statistical analysis it has been noted in the tables. P values below 0.05 were regarded as indicating a probably significant difference between means, with P values below 0.01 indicating a significant difference.

IV RESULTS

4.1. ACUTE FLUROXENE TREATMENT

4.1.1. The effects of fluroxene on hepatic microsomal enzymes and haem in induced and uninduced rats

4.1.1.1. Cytochrome P-450 and haem

The effects of fluroxene on the group of drug metabolizing enzymes known as cytochrome P-450 were determined using microsomes from uninduced and induced rats. Single injections of fluroxene into uninduced Wistar rats or Wistar rats previously induced with phenobarbital or 3-methylcholanthrene, resulted in significant decreases in the levels of hepatic microsomal cytochrome P-450 (Table 4.1). As shown in Table 4.1, the extent of loss of cytochrome P-450 in the differently treated Wistar rats decreased in the following order:

Phenobarbital induced > 3-methylcholanthrene induced
≅ uninduced (6 ml/kg) > uninduced (4 ml/kg).

In all cases where fluroxene was administered to induced or uninduced rats, the losses of cytochrome P-450 per mg of microsomal protein were accompanied by similar losses of microsomal haem per mg of microsomal protein (Table 4.1). This is consistent with earlier reports, and appears to result from the ability of fluroxene to degrade the haem moiety of cytochrome P-450 *in vivo* and *in vitro* (132). Fluroxene (4 ml/kg) did not significantly affect the levels of microsomal cytochrome P-450 or haem in Long-Evans rats *in vivo* (Table 4.1).

4.1.1.2. Haem oxygenase

The effect of fluroxene treatment in vivo on hepatic haem oxygenase was assayed in order to determine whether fluroxene had an effect on the physiological pathway for haem biodegradation.

Significant increases in the activity of hepatic microsomal haem oxygenase were observed after fluroxene treatment of uninduced Wistar rats and Wistar rats previously induced with phenobarbital or 3-methylcholanthrene (Table 4.2). In male Wistar rats the activity of haem oxygenase was increased in the following order:

Uninduced (4 ml/kg) < 3-methylcholanthrene induced
< uninduced (6 ml/kg) < phenobarbital induced.

Furthermore, fluroxene significantly affected the activity of haem oxygenase in Long-Evans rats (Table 4.2).

From the data presented in Table 4.2, it appears that there is a direct relationship between the loss of hepatic microsomal cytochrome P-450 and haem and the percentage increase in the activity of hepatic haem oxygenase following fluroxene treatment.

To determine whether haem oxygenase induction might be a consequence of the degradation of the haem of cytochrome P-450 by fluroxene, the activity of hepatic haem oxygenase and the levels of cytochrome P-450 were measured as a function of time in phenobarbital induced rats (Table 4.3). In as much as the degradation of cytochrome P-450 was observed 1 hour after fluroxene treatment, whereas the activity of haem oxygenase was not elevated at 1 hour but only at 5 hours after fluroxene treatment, it was evident that cytochrome P-450 degradation preceded the induction of hepatic haem oxygenase.

TABLE 4.1

THE EFFECTS OF FLUROXENE ON THE LEVELS OF HEPATIC MICROSOMAL CYTOCHROME P-450 AND HAEM IN INDUCED AND UNINDUCED RATS

Rats were sacrificed 24 hours after fluroxene treatment or at an equivalent time in control animals. Results are means ± S.D. for assays performed in triplicate or more on each of three or more separate groups of three animals.

INDUCTION	DOSAGE OF FLUROXENE	CYTOCHROME P-450		HAEM		LOSS HAEM/LOSS CYTOCHROME P-450
	ml/kg	nmoles/mg mic.protein	% Relative to controls	nmoles/mg mic,protein	% Relative to controls	nmoles/mg mic. protein
NONE	None	1.17±0.19		1.58±0.21		
	4.0	0.88±0.17*	75	1.46±0.30	92	0.12/0.29
NONE	None	0.85±0.14		1.49±0.13		
	6.0	0.48±0.06+	56	1.06±0.26*	71	0.43/0.37
MC	None	1.86±0.45		2.63±0.43		
	4.0	0.98±0.17+	53	1.91±0.18+	73	0.72/0.88
PB	None	1.98±0.08		2.76±0.08		
	1.0	0.73±0.01+	37	1.60±0.16+	58	1.16/1.25
[a] NONE	None	0.83±0.04		1.47±0.05		
	4.0	0.74±0.07	89	1.38±0.09	95	0.08/0.09

[a] Long Evans rats.

Abbreviations: PB, phenobarbital; MC, 3-methylcholanthrene; mic., microsomal.

* Differs significantly from identically induced rats not treated with fluroxene, $p < 0.01$.

+ Differs significantly from identically induced rats not treated with fluroxene, $p < 0.001$.

TABLE 4.2

THE EFFECTS OF FLUROXENE ON THE ACTIVITY OF HEPATIC MICROSOMAL HAEM OXYGENASE IN INDUCED AND UNINDUCED RATS

Rats were sacrificed 24 hours after fluroxene treatment or at an equivalent time in control animals. Results are means ± S.D. for assays performed in triplicate or more on each of three or more separate groups of three animals.

INDUCTION	DOSAGE OF FLUROXENE ml/kg	HAEM OXYGENASE nmoles bilirubin formed /mg mic. protein/10 min	HAEM OXYGENASE % Relative to controls	[b]CYTOCHROME P-450 % Loss relative to controls	[b]HAEM % Loss relative to controls
NONE	None	0.07±0.04			
	4.0	0.23±0.15[+]	329	25	7
NONE	None	0.09±0.03			
	6.0	0.68±0.29[‡]	756	44	29
MC	None	0.07±0.05			
	4.0	0.40±0.07[‡]	571	47	27
PB	None	0.06±0.01			
	1.0	0.51±0.09[‡]	850	63	42
[a]NONE	None	0.07±0.01			
	4.0	0.19±0.12*	271	11	5

[a] Long Evans rats. [b] From Table 4.1.

Abbreviations: mic., microsomal; MC, 3-methylcholanthrene; PB, phenobarbital.

* Probably differs from identically induced rats not treated with fluroxene, $P < 0.05$.

+ Differs significantly from identically induced rats not treated with fluroxene, $P < 0.01$.

‡ Differs significantly from identically induced rats not treated with fluroxene, $P < 0.001$.

TABLE 4.3

THE EFFECTS OF FLUROXENE ON HEPATIC CYTOCHROME P-450 AND HAEM OXYGENASE AS A FUNCTION OF TIME

All rats were induced with phenobarbital and treated or not with one fluroxene injection 24 hours later, at time zero. All rats were starved immediately after phenobarbital treatment and were sacrificed at the times after the fluroxene treatment indicated. Reported values are means ± S.D. for determinations in triplicate on each of three groups of three rats. Percentages were calculated relative to control rats not treated with fluroxene, and sacrificed at a time corresponding to 1 hour after fluroxene treatment.

HOURS AFTER FLUROXENE INJECTION	FLUROXENE	CYTOCHROME P-450		HAEM OXYGENASE	
	ml/kg	nmoles/mg mic. protein	% Relative to controls	nmoles bilirubin formed /mg mic. protein/10 min	% Relative to controls
1	None	1.27	100	0.09	100
1	1.0	0.76*	60	0.09	100
5	1.0	0.71*	66	0.63*	700
10	1.0	0.89*	70	1.21*	1344

Abbreviations: mic., microsomal.

* Differs significantly from phenobarbital induced rats not treated with fluroxene, $P < 0.001$.

4.1.2. The effects of fluroxene on the haem biosynthetic pathway in induced and uninduced rats

4.1.2.1. Urinary and faecal haem precursors

The effects of fluroxene on the haem biosynthetic pathway were assessed by measuring the amounts of haem precursors excreted in the urine and faeces after fluroxene treatment of uninduced Long Evans and Wistar rats and Wistar rats previously induced with phenobarbital or 3-methylcholanthrene.

A single injection of fluroxene into uninduced Wistar rats did not affect or resulted in a slight increase in the levels of ALA (Table 4.4). Significantly increased amounts of urinary ALA did, however, follow fluroxene treatment of 3-methylcholanthrene and phenobarbital induced rats. Porphobilinogen was significantly elevated by fluroxene treatment of uninduced and induced Wistar rats (Table 4.4). No significant changes in urinary and faecal porphyrin levels were observed after fluroxene treatment of Wistar rats (Tables 4.5 and 4.6).

Fluroxene treatment of uninduced male Long Evans rats did not result in significant increases in the levels of haem precursors in urine and faeces, except for a significant increase in the amount of ALA (Tables 4.4 - 4.6).

4.1.2.2. Hepatic ALA-synthetase

Since significantly increased levels of the urinary haem precursors porphobilinogen, and in some cases ALA, were observed following fluroxene treatment of induced and uninduced Wistar rats, the effect of fluroxene

TABLE 4.4

THE EFFECTS OF FLUROXENE ON THE LEVELS OF URINARY ALA AND PORPHOBILINOGEN IN INDUCED AND UNINDUCED RATS

Urine was collected from groups of three identically treated rats over a period of 24 hours, commencing immediately after the fluroxene injection or at an equivalent time in control rats. Results are means ± S.D. for determinations in duplicate on each of three or more groups of three rats. S.D. values for a single experiment are as given in Tables 4.11 and 4.12).

INDUCTION	DOSAGE OF FLUROXENE ml/kg	ALA µg/24 hrs/3 rats		% Relative to controls	PORPHOBILINOGEN µg/24 hrs/3 rats		% Relative to controls
NONE	None	52±24	[b](49,79,21,58)		6±2	(6,7,3,9)	
	4.0	81±29+	(114,-,73,57)	156	14±5‡	(8,-,21,12)	233
NONE	None	62±25	(72,54,90,32)		8±5	(4,15,9,5)	
	6.0	75±27	(51,107,88,54)	121	13±6*	(6,19,15,-)	163
MC	None	36±12	(24,37,47)		3±1	(3,3,5)	
	4.0	79±20+	(91,56,89)	219	26±17‡	(41,30,7)	867
PB	None	67±22	(79,-,80,41)		9±6	(3,15,8)	
	1.0	148±68‡	(249,120,96,127)	221	22±3‡	(23,24,19)	244
[a]NONE	None	91±27	(83,82,70,131)		11±4	(10,17,11,7)	
	4.0	162±60‡	(154,120,124,248)	178	9±4	(9,10,4,13)	82

[a] Long Evans rats. [b] Results obtained on individual days are given in parentheses.
Abbreviations: MC, 3-methylcholanthrene; PB, phenobarbital; ALA, δ-aminolevulinic acid.
* Statistics performed using Student's t-test for paired data, $P < 0.01$.
+ Probably differs from identically induced rats not treated with fluroxene, $P < 0.05$.
‡ Differs significantly from identically induced rats not treated with fluroxene, $P < 0.01$.
‡ Differs significantly from identically induced rats not treated with fluroxene, $P < 0.001$.

TABLE 4.5

THE EFFECTS OF FLUROXENE ON THE LEVELS OF URINARY UROPORPHYRIN AND COPROPORPHYRIN IN INDUCED AND UNINDUCED RATS

Urine was collected from groups of three identically treated rats over a period of 24 hours, commencing immediately after the fluroxene injection or an equivalent time in control rats. Results are means ± S.D. for single determinations on each of three or more groups of three rats. S.D. values for duplicate determinations on one sample are given in Tables 4.13 and 4.14.

INDUCTION	DOSAGE OF FLUROXENE ml/kg	UROPORPHYRIN µg/24 hrs/3 rats	UROPORPHYRIN % Relative to controls	COPROPORPHYRIN µg/24 hrs/3 rats	COPROPORPHYRIN % Relative to controls
NONE	None	1.6 ± 1.6		8.1 ± 4.1	
	4.0	1.9 ± 2.0	119	7.1 ± 5.8	88
NONE	None	0.8 ± 0.7		13.9 ± 3.2	
	6.0	0.8 ± 0.3	100	8.0 ± 2.0	58
MC	None	2.2 ± 0.9		25.6 ± 5.8	
	4.0	3.5 ± 0.6	159	44.5 ± 17.5	174
PB	None	1.8 ± 1.5		34.7 ± 11.7	
	1.0	2.1 ± 0.9	117	25.0 ± 10.8	72
[a] NONE	None	2.5 ± 0.2		37.6 ± 18.8	
	4.0	1.6 ± 0.6*	64	21.3 ± 13.3	57

[a] Long Evans rats. Abbreviations: MC, 3-methylcholanthrene; PB, phenobarbital.

* Probably differs from identically induced rats not treated with fluroxene, $P < 0.05$.

TABLE 4.6

THE EFFECTS OF FLUROXENE ON THE LEVELS OF FAECAL COPROPORPHYRIN AND PROTOPORPHYRIN IN INDUCED AND UNINDUCED RATS

Faeces was collected from groups of three identically treated rats over a period of 24 hours, commencing immediately after the fluroxene injection or at an equivalent time in control rats. Results are means ± S.D. for single determinations on each of three or more groups of three rats. S.D. values for duplicate determinations on one sample are given in Tables 4.15 and 4.16.

INDUCTION	DOSAGE OF FLUROXENE ml/kg	COPROPORPHYRIN µg/g dry wt./3 rats	COPROPORPHYRIN % Relative to controls	PROTOPORPHYRIN µg/g dry wt./3 rats	PROTOPORPHYRIN % Relative to controls
NONE	None	6.2 ± 2.5		35.0 ± 15.8	
	4.0	8.0 ± 3.0	129	32.8 ± 14.0	94
NONE	None	9.7 ± 5.1		43.8 ± 8.9	
	6.0	10.4 ± 3.3	107	39.8 ± 7.4	91
MC	None	9.2 ± 0.9		80.9 ± 35.0	
	4.0	10.4 ± 1.0	113	72.4 ± 27.0	89
PB	None	15.9 ± 3.5		49.6 ± 7.1	
	1.0	21.4 ± 6.7	135	74.7 ± 29.7	151
[a]NONE	None	11.3 ± 2.8		26.9 ± 7.0	
	4.0	13.0 ± 3.8	115	35.2 ± 12.9	131

[a] Long Evans Rats.

Abbreviations: MC, 3-methylcholanthrene; PB, phenobarbital; wt., weight.

on the activity of the rate-limiting enzyme of the hepatic haem biosynthetic pathway, ALA-synthetase, was measured (Table 4.7).

Both uninduced and induced Wistar rats exhibited significant increases in the activity of ALA-synthetase after fluroxene treatment. The most striking effect on the activity of ALA-synthetase was obtained with 3-methylcholanthrene induced rats.

4.2. 2,2,2-TRIFLUOROETHYL ETHYL ETHER (TFEE) TREATMENT

4.2.1. The effects of TFEE on hepatic microsomal enzymes in phenobarbital induced rats

The effects of a single injection of TFEE on hepatic microsomal enzymes were assessed in phenobarbital induced male Wistar rats (Table 4.8). As found earlier for TFEE anaesthesia (72,135), an injection of TFEE into phenobarbital induced rats did not result in the degradation of hepatic microsomal cytochrome P-450 or haem. Furthermore, TFEE did not alter the activity of hepatic microsomal haem oxygenase (Table 4.8).

4.2.2. The effects of TFEE on the levels of urinary and faecal haem precursors in phenobarbital induced rats

A single injection of TFEE into phenobarbital induced Wistar rats caused no significant alteration in the levels of the urinary or faecal haem precursors; i.e. no alteration in the levels of ALA, porphobilinogen or of the urinary or faecal porphyrins was observed (Table 4.9).

TABLE 4.7

THE EFFECTS OF FLUROXENE ON THE ACTIVITY OF HEPATIC ALA-SYNTHETASE IN INDUCED AND UNINDUCED WISTAR RATS

Rats were sacrificed 16 hours after fluroxene treatment or at an equivalent time in control animals. Results are means ± S.D. for assays performed in triplicate on each of three or more separate groups of three animals.

INDUCTION	DOSAGE OF FLUROXENE ml/kg	ALA-SYNTHETASE µg ALA formed/mg protein/30 min	ALA-SYNTHETASE % Relative to controls
NONE	None	0.024 ± 0.005	
	4.0	0.038 ± 0.006+	158
NONE	None	0.023 ± 0.004	
	6.0	0.035 ± 0.010*	152
MC	None	0.021 ± 0.004	
	4.0	0.052 ± 0.003+	248
PB	None	0.036 ± 0.004	
	1.0	0.047 ± 0.010*	131

Abbreviations: ALA, δ-aminolevulinic acid; MC, 3-methylcholanthrene; PB, phenobaribtal.

* Differs significantly from identically induced rats not treated with fluroxene, $P < 0.01$.

+ Differs significantly from identically induced rats not treated with fluroxene, $P < 0.001$.

TABLE 4.8

THE EFFECTS OF TFEE ON HEPATIC MICROSOMAL ENZYMES IN PHENOBARBITAL INDUCED RATS

Rats were sacrificed 24 hours after TFEE treatment or at an equivalent time in control animals. Results are means ± S.D. for assays performed in triplicate or more on each of three separate groups of three animals.

TFEE	CYTOCHROME P-450		HAEM		HAEM OXYGENASE	
ml/kg	nmoles/mg mic. protein	% Relative to controls	nmoles/mg mic. protein	% Relative to controls	nmoles bilirubin formed /mg mic. protein/10 min	% Relative to controls
None	1.95 ± 0.01		2.05 ± 0.02		0.04 ± 0.00	
1.0	2.04 ± 0.02	105	2.18 ± 0.09	106	0.04 ± 0.00	100

Abbreviations: TFEE, trifluoroethyl ethyl ether; mic., microsomal.

TABLE 4.9

THE EFFECTS OF TFEE ON THE LEVELS OF URINARY AND FAECAL HAEM PRECURSORS IN PHENOBARBITAL INDUCED RATS

Urine and faecal samples were collected from groups of three identically treated rats over a period of 24 hours, commencing immediately after the TFEE injection or at an equivalent time in control rats. Results are means ± S.D. for determinations on each of three groups of three rats. S.D. values for determinations in duplicate on one sample are given in Tables 4.11 - 4.16.

URINARY ASSAYS

TFEE	ALA		PORPHOBILINOGEN		UROPORPHYRIN		COPROPORPHYRIN	
ml/kg	µg/24 hrs/ 3 rats	% Relative to controls	µg/24 hrs/ 3 rats	% Relative to controls	µg/24 hrs/ 3 rats	% Relative to controls	µg/24 hrs/ 3 rats	% Relative to controls
None	58.2 ± 12.7		5.6 ± 2.0		1.8 ± 0.6		34.7 ± 11.7	
1.0	78.3 ± 20.0	135	3.9 ± 1.2	70	2.1 ± 0.9	117	25.0 ± 10.8	72

FAECAL ASSAYS

TFEE	COPROPORPHYRIN		PROTOPORPHYRIN	
ml/kg	µg/g dry weight/3 rats	% Relative to controls	µg/g dry weight/3 rats	% Relative to controls
None	11.2 ± 2.8		44.6 ± 8.9	
1.0	12.3 ± 3.8	110	40.9 ± 7.4	92

Abbreviations: TFEE, trifluoroethyl ethyl ether; ALA, δ-aminolevulinic acid.

4.3. CHRONIC FLUROXENE TREATMENT

4.3.1. The effects of fluroxene on hepatic microsomal enzymes and haem in uninduced rats

Uninduced Wistar rats were given repeated fluroxene treatment for a period of 22 days, the last injection being given 16 hours before sacrifice. Control values were taken from untreated rats kept under similar conditions for 22 days (Experimental details are given in Table 4.10).

The levels of cytochrome P-450 were unchanged relative to control values, as were the levels of hepatic microsomal haem, after chronic fluroxene treatment. In addition, the activity of hepatic haem oxygenase was unaffected after chronic fluroxene treatment (Table 4.10).

4.3.2. The effects of chronic fluroxene treatment on the hepatic haem biosynthetic pathway in uninduced rats

4.3.2.1. Urinary and faecal haem precursors

The results of two separate experiments showing the effects of chronic fluroxene treatment on the levels of urinary and faecal haem precursors are reported in Tables 4.11 - 4.16. In both experiments, day 1 - where no fluroxene had as yet been administered to the rats - was treated as the control. Identical control values were obtained with respect to levels of haem precursors, from rats given no fluroxene but kept under the same conditions as the experimental rats for the duration of the experiment (data not shown). The results from each experiment represent assays on a group of 3 rats given 1 ml/kg of fluroxene every 48 hours.

TABLE 4.10

THE EFFECTS OF CHRONIC FLUROXENE TREATMENT ON HEPATIC MICROSOMAL ENZYMES AND HAEM IN UNINDUCED RATS

Rats were injected with fluroxene at a dose of 1 ml/kg every 48 hours for a period of 22 days (10 injections of fluroxene). Animals were sacrificed on day 23, 16 hours after the final fluroxene injection or at an equivalent time in control animals. Control values were taken from untreated rats kept under identical conditions for the duration of the experiment. Results are means ± S.D. for determinations in triplicate on each of two or more groups of three rats.

NO. OF INJECTIONS	CYTOCHROME P-450		HAEM		HAEM OXYGENASE	
	nmoles/mg mic. protein	% Relative to controls	nmoles/mg mic. protein	% Relative to controls	nmoles bilirubin formed /mg mic. protein/10 min	% Relative to controls
None	1.11 ± 0.28		1.81 ± 0.17		0.114 ± 0.03	
10	1.08 ± 0.23	97	1.71 ± 0.44	94	0.125 ± 0.04	110

Abbreviation : mic., microsomal.

Experiment 1 was performed over a period of 16 days (7 injections of fluroxene) and experiment 2 was continued for a total of 22 days (10 injections of fluroxene).

In both experiments, the levels of urinary ALA were generally increased following chronic fluroxene treatment, but a dose-response relationship was not evident (Table 4.11). Urinary porphobilinogen levels generally increased with an increasing number of injections of fluroxene over the experimental period. Extremely high levels of porphobilinogen were found in the urine of experimental animals towards the end of both experiments 1 and 2 (Table 4.12); increases corresponding to ca. 2000% of the levels of porphobilinogen in the urine of control rats were observed.

Urinary uroporphyrin levels were initially increased in both experiments but gradually decreased in experiment 2 until the levels were equivalent to the control value or below (Table 4.13). Coproporphyrin levels in the urine were slightly increased after one fluroxene injection and remained elevated throughout the experiments (Table 4.14). The faeces of the rats studied showed slightly decreased levels of both coproporphyrin and protoporphyrin relative to day 1 of the experiments (Tables 4.15 and 4.16).

4.3.2.2. Hepatic ALA-synthetase

The activity of the rate-limiting enzyme of haem biosynthesis, ALA-synthetase, was significantly elevated after chronic treatment of rats with fluroxene for a period of 22 days (Table 4.17). Control values were measured using untreated rats kept under similar conditions for the duration of the experiment (Details are given in Table 4.17).

TABLE 4.11

THE EFFECTS OF CHRONIC FLUROXENE TREATMENT ON THE LEVELS OF URINARY ALA IN UNINDUCED RATS

Uninduced rats were injected with fluroxene (1.0 ml/kg) every 48 hours. Urine was collected from groups of three rats over a period of 48 hours, commencing immediately after each injection of fluroxene. Day 1 - where no fluroxene had as yet been administered to the rats - was treated as the control. Results are means for determinations in duplicate on each separate group of three rats. The S.D. value for duplicate determinations on one sample is 1.8.

DAY	No. OF FLUROXENE INJECTIONS	ALA					
		EXPERIMENT 1		EXPERIMENT 2		AVERAGE	
		µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls
1	0	55.2	100	60.0	100	57.6	100
3	1	120.0	217	89.7	150	104.9^{+}	182
5	2	67.2	122	75.0	125	71.1*	123
7	3	47.7	86	78.0	130	62.9	109
9	4	57.6	104	73.2	122	65.4	114
11	5	64.8	117	98.1	164	81.5^{+}	141
13	6	102.6	186	70.8	118	86.7^{+}	151
15	7	92.4	167	45.3	76	68.9*	120
17	8			72.0	120		
19	9			89.4	149		
21	10			162.0	270		

Abbreviation: ALA, δ-aminolevulinic acid. * Probably differs from control rats, $P < 0.05$.

$^{+}$ Differs significantly from control rats, $P < 0.01$.

TABLE 4.12

THE EFFECTS OF CHRONIC FLUROXENE TREATMENT ON THE LEVELS OF URINARY PORPHOBILINOGEN IN UNINDUCED RATS

Details as in Table 4.11. The S.D. value for duplicate determinations on one sample is 0.9.

DAY	NO. OF FLUROXENE INJECTIONS	PORPHOBILINOGEN					
		EXPERIMENT 1		EXPERIMENT 2		AVERAGE	
		µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls
1	0	1.5	100	5.7	100	3.6	100
3	1	1.2	80	7.2	126	4.2	117
5	2	11.4	760	21.6	379	16.5*	458
7	3	9.3	620	13.8	242	11.6*	322
9	4	11.1	740	13.2	232	12.2*	339
11	5	18.0	1200	16.5	289	17.3*	481
13	6	30.6	2040	32.7	574	31.7+	881
15	7	34.5	2300	24.0	421	29.3*	814
17	8			30.6	537		
19	9			36.0	632		
21	10			96.3	1689		

* Differs significantly from control rats, $P < 0.01$.

+ Differs significantly from control rats, $P < 0.001$.

TABLE 4.13

THE EFFECTS OF CHRONIC FLUROXENE TREATMENT ON THE LEVELS OF URINARY UROPORPHYRIN IN UNINDUCED RATS

Details as in Table 4.11. Results are single determinations on each separate group of three rats. The S.D. value for duplicate determinations on one sample is 0.5.

DAY	NO. OF FLUROXENE INJECTIONS	UROPORPHYRIN					
		EXPERIMENT 1		EXPERIMENT 2		AVERAGE	
		µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls
1	0	0.5	100	2.4	100	1.5	100
3	1	0.8	160	9.3	388	5.1	340
5	2	1.2	240	6.3	263	3.8	253
7	3	1.1	220	4.5	188	2.8	187
9	4	1.3	260	2.4	100	1.9	127
11	5	1.3	260	5.1	213	3.2	213
13	6	1.7	340	2.4	100	2.1	140
15	7	1.2	240	0.6	25	0.9	60
17	8			0.9	38		
19	9			2.1	88		
21	10			2.1	88		

TABLE 4.14

THE EFFECTS OF CHRONIC FLUROXENE TREATMENT ON THE LEVELS OF URINARY COPROPORPHYRIN IN UNINDUCED RATS

Details as in Table 4.11. Results are single determinations on each separate group of three rats. The S.D. value for duplicate determinations on one sample is 4.5.

DAY	NO. OF FLUROXENE INJECTIONS	COPROPORPHYRIN					
		EXPERIMENT 1		EXPERIMENT 2		AVERAGE	
		µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls
1	0	7.8	100	13.2	100	10.5	100
3	1	18.6	238	26.7	202	22.7	216
5	2	19.2	246	43.8	332	31.5	300
7	3	14.4	185	27.6	209	21.0	200
9	4	19.8	254	19.5	148	19.7	188
11	5	15.6	200	27.6	209	21.6	206
13	6	30.3	388	18.3	116	24.3	231
15	7	21.6	277	24.3	184	23.0	219
17	8			24.9	189		
19	9			35.1	266		
21	10			33.0	250		

TABLE 4.15

THE EFFECTS OF CHRONIC FLUROXENE TREATMENT ON THE LEVELS OF FAECAL COPROPORPHYRIN IN UNINDUCED RATS

Uninduced rats were injected with fluroxene (1.0 ml/kg) every 48 hours. Faeces was collected from groups of three rats over a period of 48 hours, commencing immediately after each injection of fluroxene.

Day 1 - where no fluroxene had as yet been administered to the rats - was treated as the control. Results are single determinations on each separate group of three rats. The S.D. value for duplicate determinations on one sample is 4.0.

DAY	NO. OF FLUROXENE INJECTIONS	COPROPORPHYRIN			
		EXPERIMENT 1		EXPERIMENT 2	
		µg/g dry wt. /3 rats	% Relative to controls	µg/g dry wt. /3 rats	% Relative to controls
1	0	21.6	100	14.7	100
3	1	9.0	42	10.8	73
5	2			12.3	84
7	3			14.4	98
9	4			10.5	71
11	5			11.7	80
13	6			10.5	71
15	7	13.2	61		
17	8			8.7	59
19	9			6.9	47
21	10			7.8	53

Abbreviation: wt., weight.

TABLE 4.16

THE EFFECTS OF CHRONIC FLUROXENE TREATMENT ON THE LEVELS OF FAECAL PROTOPORPHYRIN IN UNINDUCED RATS

Details as in Table 4.15. Results are single determinations on each group of three rats. The S.D. value for duplicate determinations on one sample is 8.5.

DAY	NO. OF FLUROXENE INJECTIONS	PROTOPORPHYRIN			
		EXPERIMENT 1		EXPERIMENT 2	
		µg/g dry wt. /3 rats	% Relative to controls	µg/g dry wt. /3 rats	% Relative to controls
1	0	53.1	100	41.7	100
3	1	24.3	46	36.9	88
5	2			41.1	99
7	3			50.7	122
9	4			31.8	76
11	5			36.6	88
13	6			34.2	82
15	7	38.7	73		
17	8			40.2	96
19	9			31.8	76
21	10			31.8	76

Abbreviation: wt., weight.

TABLE 4.17

THE EFFECTS OF CHRONIC FLUROXENE TREATMENT ON THE ACTIVITIES OF HEPATIC ALA-SYNTHETASE AND UROPORPHYRINOGEN SYNTHETASE IN UNINDUCED RATS

Animals were injected with fluroxene at a dose of 1 ml/kg every 48 hours for a period of 22 days (10 injections of fluroxene). Rats were sacrificed on day 23, 16 hours after the final fluroxene injection, or at an equivalent time in control animals. Control values were taken from untreated rats kept under identical conditions for the duration of the experiment. Results are means ± S.D. for determinations in triplicate on each of two or more groups of three rats.

FLUROXENE	ALA-SYNTHETASE		UROPORPHYRINOGEN SYNTHETASE	
ml/kg	µg ALA formed /mg protein /30 min	% Relative to controls	µg PBG used /mg protein /60 min	% Relative to controls
None	0.021±0.003		2.95±0.35	
1.0	0.042±0.002+	200	1.35±0.07*	46

Abbreviations: PBG, porphobilinogen; ALA, δ-aminolevulinic acid.

* Differs significantly from identically treated rats not receiving fluroxene, $P < 0.01$.

+ Differs significantly from identically treated rats not receiving fluroxene, $P < 0.001$.

4.3.2.3. Hepatic uroporphyrinogen synthetase

Since the levels of porphobilinogen, and to a lesser extent ALA levels, were elevated in the urines of uninduced rats after chronic fluroxene treatment, the activity of hepatic uroporphyrinogen synthetase, the enzyme which catalyzes the formation of uroporphyrinogen from porphobilinogen (Fig. 1.11) was determined in rats after chronic fluroxene treatment for a period of 22 days. The activity of uroporphyrinogen synthetase in these animals was approximately 50% of that found in control animals (Table 4.17).

4.4. FLUROXENE TREATMENT OF DDC TREATED RATS

4.4.1. The effect of fluroxene on the activity of hepatic ALA-synthetase in DDC treated rats

DDC (100 mg/kg in oil) and fluroxene (4 ml/kg) were injected into a group of Wistar rats, with the control animals receiving DDC alone. In these animals, hepatic ALA-synthetase activity was determined using a sensitive isotopic assay (269) capable of detecting small changes in the activity of ALA-synthetase. The activity of ALA-synthetase was found to be slightly (and probably significantly) increased in fluroxene treated rats relative to the control animals (Table 4.18).

4.5. CHARACTERIZATION OF THE GREEN PIGMENT

When the livers of rats were treated with fluroxene *in vivo* or *in vitro*, a green pigment (or haem adduct) collected in the microsomal fraction of the liver (132). Isolation and purification of the green pigment using HPLC or Sephadex LH-20 chromatography, as described in Section 3.8,

TABLE 4.18

THE EFFECT OF FLUROXENE TREATMENT ON THE ACTIVITY OF HEPATIC ALA-SYNTHETASE IN DDC TREATED RATS

Rats were sacrificed 16 hours after fluroxene treatment or at an equivalent time in control animals. Results are means ± S.D. for determinations in triplicate on three or more rats.

FLUROXENE	ALA-SYNTHETASE	
ml/kg	μmoles ALA formed/g wet wt. of liver/60 min	% Relative to controls
None	1.9 ± 0.4	
4.0	2.6 ± 0.3*	137

Abbreviations: ALA, δ-aminolevulinic acid; DDC, dicarbethoxydihydrocollidine; wt., weight.

* Probably differs from identically induced rats not treated with fluroxene, $P < 0.05$.

gave rise to three distinct fractions of material with absorbance maxima around 400 nm: a brown fraction containing haem, a fraction with a typical porphyrin spectrum containing protoporphyrin IX and a green fraction containing the adduct. The green pigment was found to be distinct from both protoporphyrin IX and haem when analyzed by absorption spectroscopy (Figs. 4.1, 4.2). Furthermore, when chromatographed on TLC plates (Section 3.8.1), the green pigment was clearly separable from both the haem and protoporphyrin IX fractions - approximate R_f values are: green pigment methyl ester, 0.21; haem methyl ester, 0.36; protoporphyrin IX methyl ester, 0.80.

The scarlet-red fluorescence of the green pigment under U.V. light and the characteristics of its absorption spectrum indicated that the green pigment was a porphyrin derivative. A neutral aetio-type porphyrin spectrum was obtained after isolation and methylation of the adduct generated in vitro (Fig. 4.1). When the adduct was isolated using Sephadex LH-20 chromatography (Section 3.8.2), the green fraction showed a typical porphyrin dication spectrum (peaks at 416 nm, 562 nm, 608 nm) (238).

In the absorption spectrum of the adduct at neutrality, the absorbance maxima were shifted towards the red compared with those of protoporphyrin IX (Fig. 4.1); the observed shifts were very similar to those previously reported for N-methyl octaethyl porphyrin and for other N-substituted green pigments (131, 280). Furthermore, the zinc complex of the methylated adduct showed an unusual bathochromic shift of the Soret band from 413 nm to 430 nm (zinc complex) (Fig. 4.1). This has previously been demonstrated for N-methylated octaethylporphyrin and is characteristic of an N-substituted porphyrin.

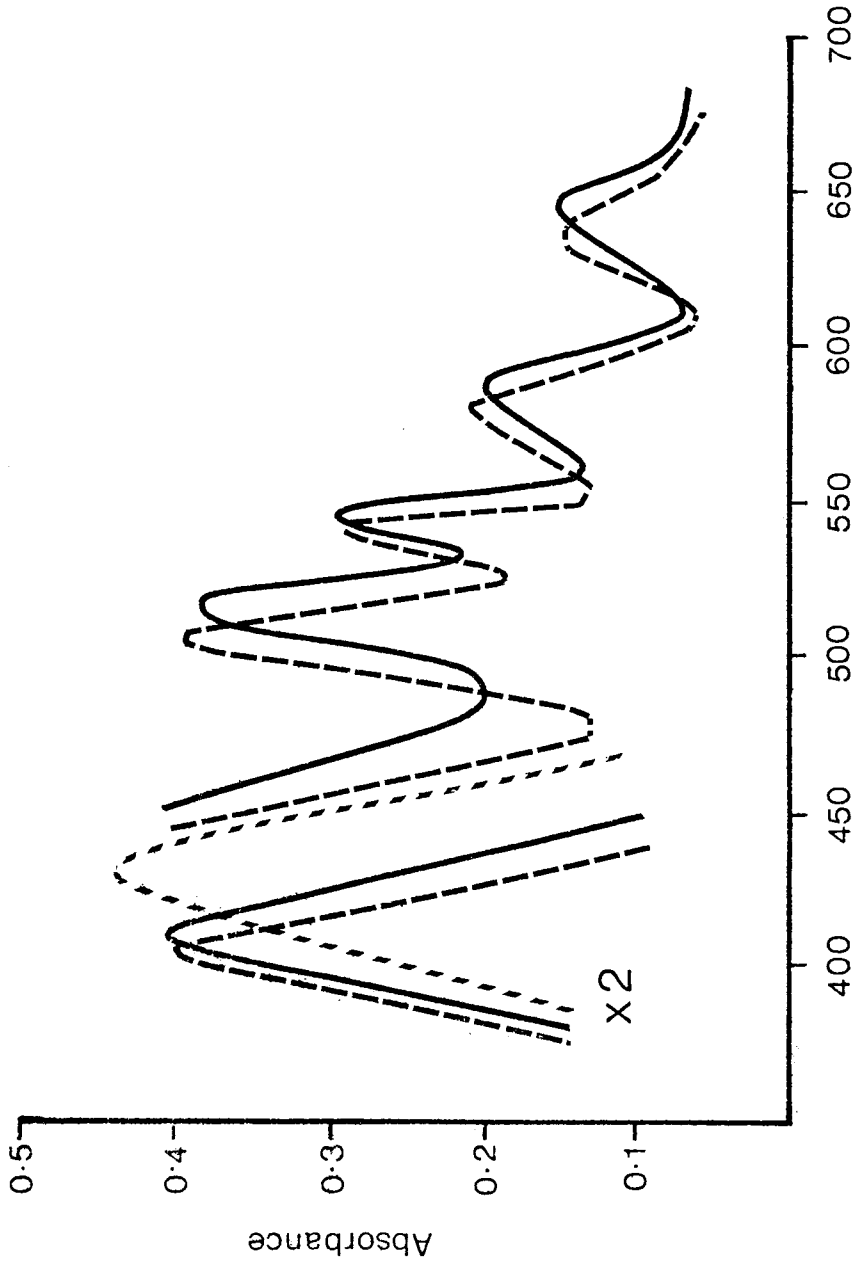


FIGURE 4.1. ABSORPTION SPECTRUM OF THE GREEN PIGMENT (METHYL ESTER) : COMPARISON WITH THE SPECTRUM OF PROTOPORPHYRIN IX (METHYL ESTER) : SORET BAND OF THE ZINC COMPLEX OF THE GREEN PIGMENT (METHYL ESTER).

The complete visible absorption spectrum of the green pigment obtained from incubation of fluroxene with hepatic microsomes in vitro (Section 3.8.1) (——) is compared with that of protoporphyrin IX (---) (both spectra run in chloroform). The bathochromic shift of the Soret band of the zinc complex of the adduct is also shown (••••). Note that the scale of the ordinates has been increased in the Soret region by a factor of 2.

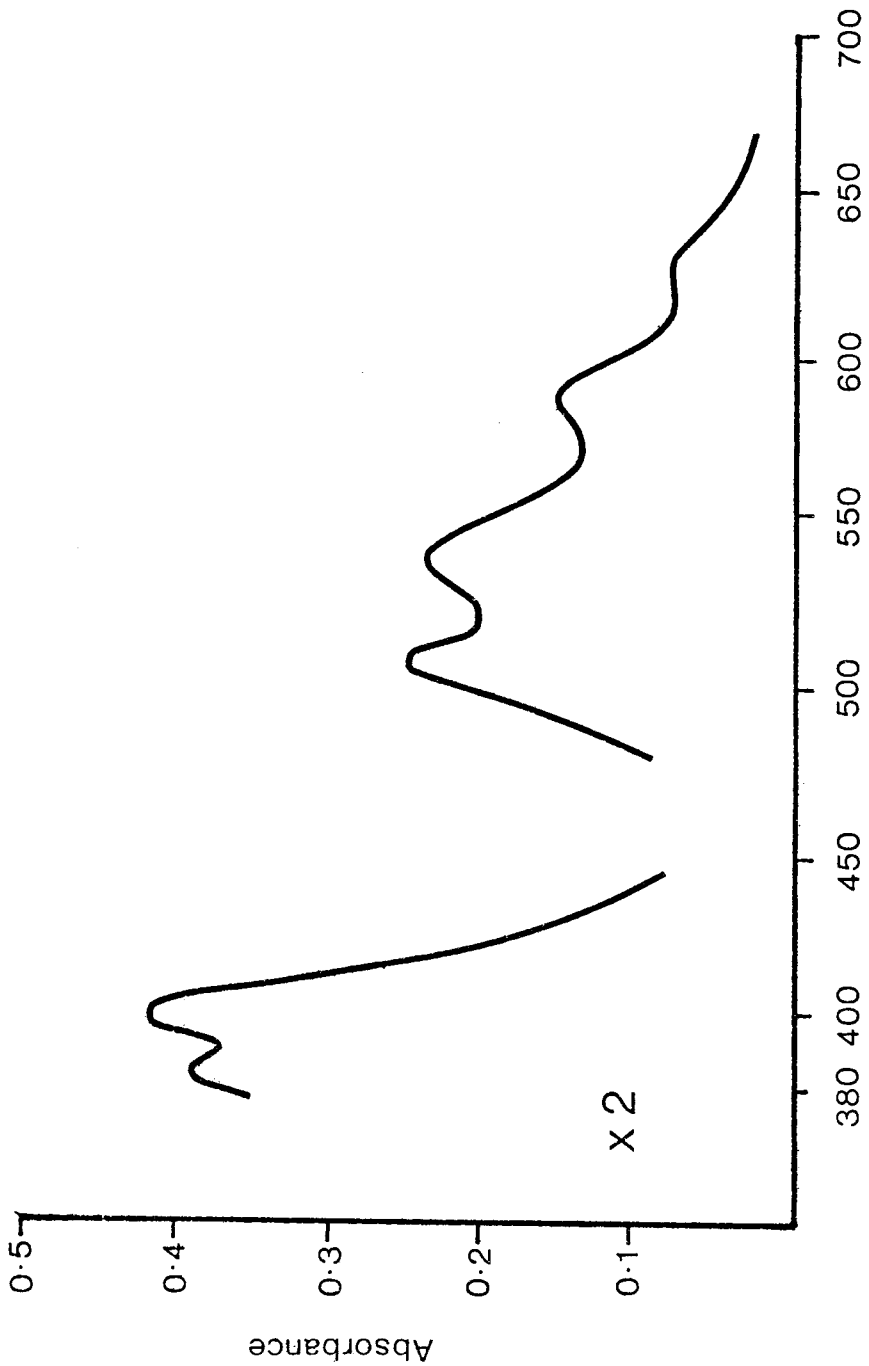


FIGURE 4.2. ABSORPTION SPECTRUM OF HAEM (METHYL ESTER)

The visible absorption spectrum of haem (methyl ester) (run in chloroform). Note that the scale of the ordinates has been increased in the Soret region by a factor of 2.

(The substituent on the pyrrole nitrogen results in steric strain on the molecule and probable incomplete co-ordination of the metal) (237).

The above facts indicated that the green pigment was an N-substituted porphyrin.

Further characterization of the adduct required mass spectroscopy and nuclear magnetic resonance using highly sophisticated equipment to which I had no access. Attempts to transport the isolated adduct to San Francisco, USA, for further characterization failed due to the instability of the adduct (281).

Kunze et al. (138), using field desorption mass spectroscopy and 360 mHz NMR spectroscopy, confirmed that the adduct is an N-substituted porphyrin - the exact structure of the adduct being N-(2-oxoethyl)proto-porphyrin IX.

4.6. THE ACTIVITY OF HEPATIC UROPORPHYRINOGEN SYNTHETASE IN VITRO

4.6.1. The effects of fluroxene and its metabolites on the activity of hepatic uroporphyrinogen synthetase in vitro

The activity of hepatic uroporphyrinogen synthetase was inhibited by 50% after chronic fluroxene treatment in vivo (Table 4.17). In order to determine whether fluroxene or its metabolites viz. 2,2,2-trifluoroethanol, trifluoroacetic acid and trifluoroacetaldehyde were responsible for the observed decrease in the activity of uroporphyrinogen synthetase, the

effects of these compounds on the activity of hepatic uroporphyrinogen synthetase were determined *in vitro* (details are given in Table 4.19). As shown in Table 4.19, neither fluroxene nor its metabolites had any significant effect on the activity of uroporphyrinogen synthetase *in vitro*.

4.6.2. The effect of the green pigment on the activity of uroporphyrinogen synthetase *in vitro*

Since fluroxene and its metabolites caused no significant change in the activity of uroporphyrinogen synthetase *in vitro* (Table 4.19), the effect of the green pigment on the activity of uroporphyrinogen synthetase was determined *in vitro* (Table 4.20). The green pigment was isolated in acetone : HCl (120 : 0.1 (v/v)), and neutralized with 10 M KOH; therefore acetone, acetone HCl (120:0.1 (v/v) (neutralized)) and 3 M KCl were utilized as controls. From Table 4.20 it is evident that the green pigment had no significant effect on the activity of hepatic uroporphyrinogen synthetase *in vitro* under the conditions of our experiments.

4.7. ACUTE HALOTHANE TREATMENT *IN VIVO*

4.7.1. The effects of halothane on hepatic microsomal enzymes and haem in induced and uninduced rats 24 hours after acute halothane treatment

4.7.1.1. Cytochrome P-450 and haem.

The effect of a single injection of halothane on the levels of hepatic cytochrome P-450 and haem in Wistar rats treated or not with phenobarbital

TABLE 4.19

THE EFFECTS OF FLUROXENE AND ITS METABOLITES ON THE ACTIVITY OF UROPORPHYRINOGEN SYNTHETASE IN VITRO

Uroporphyrinogen synthetase in post-mitochondrial supernatant from untreated rats (Section 3.5.2) was incubated with PBG (0.05 µmoles) and additions as indicated below, at 37°C for 60 min. The activity of uroporphyrinogen synthetase was determined as described in Section 3.5.2. The standard reaction mixture contained post-mitochondrial supernatant and PBG (0.05 µmoles) (266).

ADDITIONS (0.5 mM final conc.)	UROPORPHYRINOGEN SYNTHETASE ACTIVITY	
	µg PBG used/mg protein /60 min	% Relative to standard
None	0.013±0.003	100
Fluroxene	0.015±0.001	115
2,2,2-Trifluoroethanol	0.012±0.003	92
Trifluoroacetic Acid	0.012±0.003	92
Trifluoroacetaldehyde	0.015±0.003	115

Abbreviations: PBG, porphobilinogen; conc., concentration.

TABLE 4.20

THE EFFECTS OF THE GREEN PIGMENT ON THE ACTIVITY OF UROPORPHYRINOGEN SYNTHETASE IN VITRO

The green pigment was isolated in acetone:HCl (120:0.1 (v/v)) and purified using Sephadex LH-20 chromatography as described in Section 3.8.2. Acetone: HCl (120:0.1 (v/v)) and the purified green pigment were neutralized with 10 M KOH prior to their incubation with uroporphyrinogen synthetase. Uroporphyrinogen synthetase in post-mitochondrial supernatant from untreated rats (Section 3.5.2) was incubated with PBG (0.05 µmoles) and the neutralized green pigment or controls as indicated below. The activity of uroporphyrinogen synthetase was calculated as described in Section 3.5.2. The standard reaction mixture contained post-mitochondrial supernatant and PBG (0.05 µmoles) (266).

ADDITIONS 100 µl	UROPORPHYRINOGEN SYNTHETASE ACTIVITY	
	µg PBG used/mg protein /60 min	% Relative to standard
None	0.014±0.003	100
Acetone	0.015±0.001	107
Acetone/HCl (120:0.1 (v/v))	0.018±0.003	129
KCl (3 M)	0.016±0.001	114
Green pigment	0.017±0.004	121

Abbreviation: PBG, porphobilinogen.

is shown in Table 4.21. The levels of cytochrome P-450 were significantly decreased in both phenobarbital induced and uninduced rats 24 hours after halothane treatment. Although the losses of cytochrome P-450 in nmol/mg of microsomal protein were greater in phenobarbital induced rats than in rats not pretreated with this inducing agent, the percentage loss of cytochrome P-450 following halothane treatment was identical in phenobarbital induced and uninduced rats. Although the losses of cytochrome P-450 were accompanied by similar losses of microsomal haem (per mg of microsomal protein) ($P > 0.05$) in both untreated and phenobarbital pretreated rats, small but variable amounts of cytochrome P-420 were observed in the microsomes of uninduced and phenobarbital induced rats after halothane treatment in vivo (Table 4.21) (See also Tables 4.24, 4.25).

4.7.1.2. Haem oxygenase.

Significant increases in the activity of hepatic haem oxygenase were observed 24 hours after halothane treatment of uninduced and phenobarbital induced rats; the effect of halothane on haem oxygenase activity was more striking in uninduced than in phenobarbital induced rats (Table 4.21).

4.7.2. The effects of halothane on hepatic haem biosynthesis in induced and uninduced rats after acute halothane treatment

The effect of acute halothane treatment in vivo on hepatic haem biosynthesis was assessed by determining its effects on the activity of hepatic ALA-synthetase and on the levels of urinary haem precursors. When assayed 24 hours after acute halothane treatment, no significant change in the activity of hepatic ALA-synthetase was observed in

TABLE 4.21

THE EFFECTS OF HALOTHANE IN VIVO ON HEPATIC MICROSOMAL ENZYMES AND HAEM IN PHENOBARBITAL INDUCED AND UNINDUCED RATS

Rats were sacrificed 24 hours after halothane treatment or at an equivalent time in control animals. Results are means ± S.D. for assays performed in triplicate on each of three or more separate groups of three animals.

INDUCTION	DOSAGE OF HALOTHANE ml/kg	CYTOCHROME P-450 nmoles/mg mic. protein	% Relative to controls	HAEM nmoles/mg mic. protein	% Relative to controls	LOSS HAEM/LOSS CYTOCHROME P-450 nmoles/mg mic. protein	HAEM OXYGENASE nmoles bilirubin formed/mg mic. protein/10 min.	% Relative to controls
NONE	None	1.03 ± 0.16		1.88 ± 0.24			0.13 ± 0.03	
	2.0	0.65 ± 0.19*+	63	1.43 ± 0.22*	76	0.45/0.38	0.65 ± 0.21*	513
PB	None	2.65 ± 0.45		3.75 ± 0.73			0.11 ± 0.04	
	1.0	1.67 ± 0.29*+	63	2.75 ± 0.35*	73	1.00/0.98	0.26 ± 0.01*	229

Abbreviations: mic., microsomal; PB, phenobarbital.

* Differs significantly from value for identically induced rats not receiving halothane, $P < 0.01$.

+ Cytochrome P-420 present.

phenobarbital induced or uninduced rats (Table 4.22).

In uninduced rats acute halothane treatment resulted in a significant increase in the levels of urinary ALA and porphobilinogen (Table 4.23) but did not alter the levels of the urinary porphyrins (Table 4.23). Furthermore, acute halothane treatment did not affect the levels of any of the urinary haem precursors in phenobarbital induced rats over the 24 hour collection period (Table 4.23).

4.7.3. The effects of acute halothane treatment on hepatic enzymes and haem as a function of time in phenobarbital induced rats in vivo

4.7.3.1. Cytochrome P-450 and haem.

In these experiments phenobarbital induced rats were injected with halothane at time zero and sacrificed at 1, 5 and 10 hours after halothane treatment. Phenobarbital induced rats which did not receive halothane were utilized as the controls, and were sacrificed at times corresponding to 0, 5 and 10 hours after halothane treatment.

One hour after the acute halothane treatment of phenobarbital induced rats, a significant decrease in the levels of microsomal cytochrome P-450 and haem were observed (Table 4.24). The losses of cytochrome P-450 and haem were maintained at both 5 and 10 hours after halothane treatment (relative to each corresponding set of controls). Generally at each time assayed the extent of loss of hepatic microsomal cytochrome P-450 was equivalent to the extent of loss of hepatic microsomal haem ($P > 0.05$) (Table 4.24).

Acute halothane treatment was found to result in the production of

TABLE 4.22

THE EFFECTS OF HALOTHANE IN VIVO ON THE ACTIVITY OF HEPATIC ALA-SYNTHETASE IN PHENOBARBITAL INDUCED AND UNINDUCED RATS

Rats were sacrificed 24 hours after halothane treatment or at an equivalent time in control animals. Results are means ± S.D. for assays performed in triplicate on each of three or more separate groups of three animals.

INDUCTION	DOSAGE OF HALOTHANE ml/kg	ALA-SYNTHETASE μg ALA formed/mg protein/30 min	ALA-SYNTHETASE % Relative to controls
NONE	None	0.017 ± 0.003	
	2.0	0.013 ± 0.006	76
PB	None	0.014 ± 0.006	
	1.0	0.015 ± 0.005	107

Abbreviations: ALA, δ-aminolevulinic acid; PB, phenobarbital.

TABLE 4.23

THE EFFECTS OF HALOTHANE IN VIVO ON THE LEVELS OF URINARY HAEM PRECURSORS IN PHENOBARBITAL INDUCED AND UNINDUCED RATS

Urine was collected from groups of three identically treated rats over a period of 24 hours, commencing immediately after the halothane injection or at an equivalent time in control rats. Results are means ± S.D. for determinations on each of three or more groups of three rats. S.D. values for determinations in duplicate on one sample are given in Tables 4.28 - 4.31.

INDUCTION	DOSAGE OF HALOTHANE ml/kg	ALA µg/24 hrs /3 rats	ALA % Relative to controls	PORPHOBILINOGEN µg/24 hrs /3 rats	PORPHOBILINOGEN % Relative to controls	UROPORPHYRIN µg/24 hrs /3 rats	UROPORPHYRIN % Relative to controls	COPROPORPHYRIN µg/24 hrs /3 rats	COPROPORPHYRIN % Relative to controls
NONE	None	52 ± 13		6 ± 4		4.0 ± 3.8		10 ± 8	
	2.0	73 ± 27*	140	13 ± 7*	217	3.6 ± 2.3	90	10 ± 4	97
PB	None	88 ± 13		26 ± 12		3.1 ± 1.7		23 ± 14	
	1.0	100 ± 13	114	22 ± 7	85	1.8 ± 0.4	58	19 ± 14	85

Abbreviations: PB, phenobarbital; ALA, δ-aminolevulinic acid.

* Differs significantly from identically induced rats not treated with halothane, using the Student's t-test for paired data, $P < 0.01$.

TABLE 4.24

THE EFFECTS OF HALOTHANE *IN VIVO* ON THE LEVELS OF HEPATIC MICROSOMAL CYTOCHROME P-450 AND HAEM AS A FUNCTION OF TIME IN PHENOBARBITAL INDUCED RATS

All rats were induced with phenobarbital and treated or not with one halothane injection 24 hours later at time zero. All rats were starved immediately after phenobarbital treatment. Rats were sacrificed at the times after the halothane treatment indicated or at an equivalent time in controls. Results are means ± S.D. for determinations in triplicate on two or more separate groups of three animals.

HOURS AFTER HALOTHANE INJECTION	DOSAGE OF HALOTHANE ml/kg	CYTOCHROME P-450 nmoles/mg mic. protein	CYTOCHROME P-450 % Relative to controls	HAEM nmoles/mg mic. protein	HAEM % Relative to controls	LOSS HAEM/LOSS OF CYTOCHROME P-450 nmoles/mg mic. protein
0	0	1.95 ± 0.04		2.98 ± 0.44		
1	1.0	1.61 ± 0.07+‡	83	2.55 ± 0.28*	86	0.43/0.34
5	0	2.16 ± 0.07		2.93 ± 0.20		
5	1.0	1.87 ± 0.13+‡	87	2.70 ± 0.04*	92	0.23/0.29
10	0	2.32 ± 0.10		2.96 ± 0.09		
10	1.0	1.96 ± 0.15+‡	84	2.71 ± 0.08+	92	0.25/0.36

Abbreviations: mic., microsomal.
* Probably differs from identically treated rats not receiving halothane, $P < 0.05$.
+ Differs significantly from identically treated rats not receiving halothane, $P < 0.01$.
‡ Cytochrome P-420 present (see Table 4.25, Fig. 4.3).

hepatic microsomal cytochrome P-420 *in vivo* (Tables 4.21, 4.24, 4.25). In order to obtain an estimate of the amount of cytochrome P-420 produced (in arbitrary units) at each time point studied (Table 4.25), the method of calculation described in Fig. 4.3 was utilized. A significant increase in theproduction of cytochrome P-420 was observed at 5 hours and 10 hours after acute halothane treatment *in vivo*, relative to the corresponding control (Table 4.25). However, at these time points (*viz.* at 5 and 10 hours after halothane treatment) the loss of hepatic microsomal haem was equivalent to the loss of hepatic microsomal cytochrome P-450 ($P < 0.05$) (Table 4.24). Therefore, it was presumed that the amount of cytochrome P-420 produced after acute halothane treatment *in vivo* was too small to cause a significant difference between the losses of cytochrome P-450 and haem.

4.7.3.2. Haem oxygenase.

The activity of hepatic haem oxygenase was significantly increased at 1, 5 and 10 hours after acute halothane treatment (relative to each corresponding set of control animals) (Table 4.26). At 5 hours after acute halothane treatment, the activity of hepatic haem oxygenase was significantly increased relative to its activity 1 hour after acute halothane treatment ($P < 0.01$). No further significant increase in haem oxygenase activity was observed between 5 hours and 10 hours after acute halothane treatment ($P > 0.1$).

4.7.3.3. ALA-synthetase.

The effect of acute halothane treatment as a function of time on the activity of ALA-synthetase in phenobarbital induced rats was most striking. One hour after acute halothane treatment, the activity of

TABLE 4.25

THE EFFECTS OF HALOTHANE IN VIVO ON THE PRODUCTION OF CYTOCHROME P-420 AS A FUNCTION OF TIME IN PHENOBARBITAL INDUCED RATS

Details as in Table 4.24. Results are means for determinations in triplicate on each separate group of three animals. Individual values were calculated as described in Fig. 4.3.

HOURS AFTER HALOTHANE INJECTION	DOSAGE OF HALOTHANE ml/kg	CYTOCHROME P-420			
		EXPERIMENT 1	EXPERIMENT 2	AVERAGE	
		ΔA*	ΔA*	ΔA*	Increase relative to controls ΔA*
0	0	0.042	0.064	0.053 ±.014	
1	1.0	0.068	0.077	0.073 ±.011	0.020
5	0	0.049	0.040	0.045 ±.008	
5	1.0	0.100	0.120	0.110 ±.021	0.065‡
10	0	0.041	0.030	0.036 ±.013	
10	1.0	0.056	0.102	0.079 ±.028	0.043+

*ΔA = Absorbance units, calculated as described in Fig. 4.3.

+ Probably differs from identically treated rats not receiving halothane, $P < 0.05$.

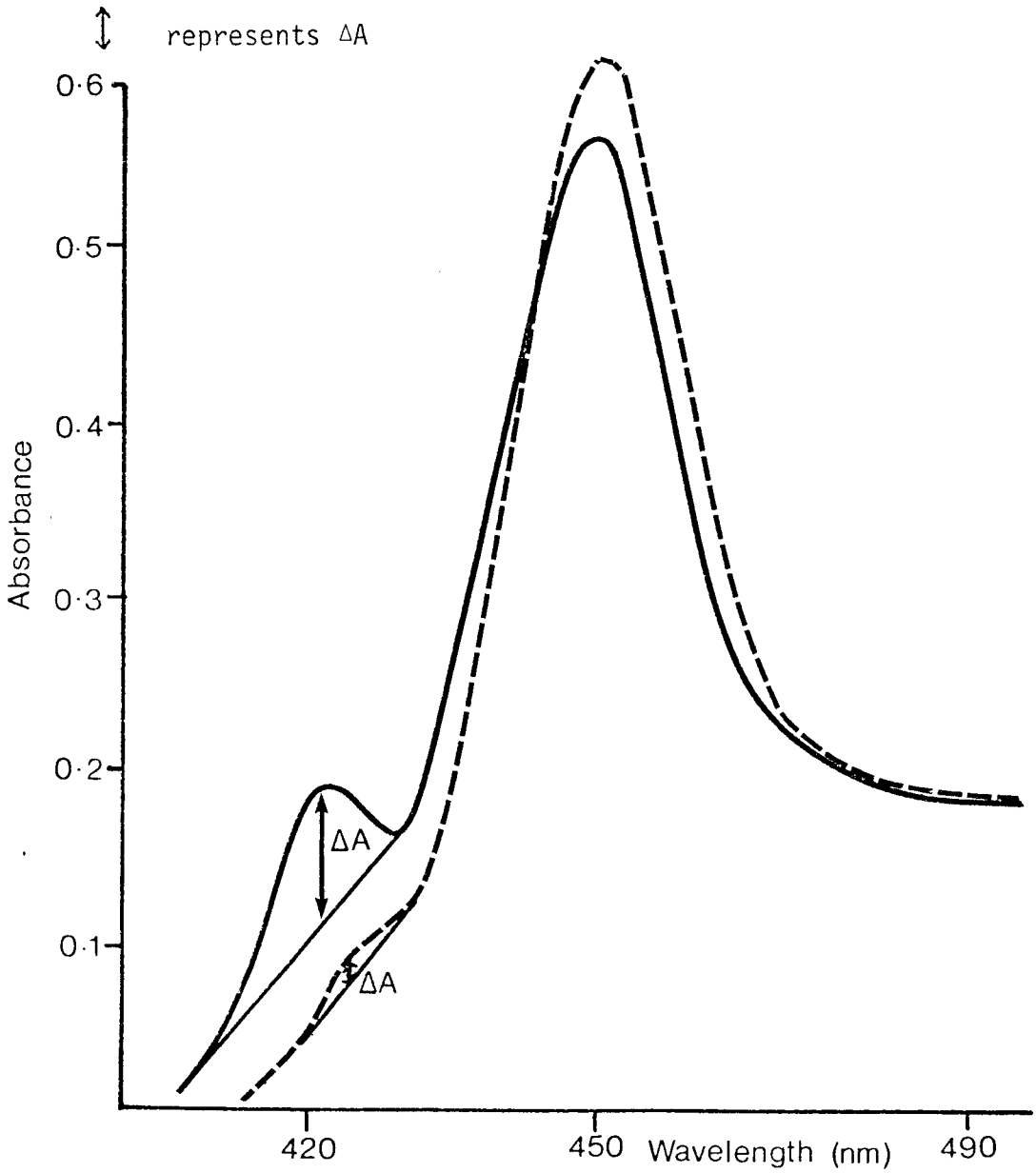
‡ Differs significantly from identically treated rats not receiving halothane, $P < 0.01$.

FIGURE 4.3

THE EFFECT OF HALOTHANE TREATMENT ON THE PRODUCTION OF CYTOCHROME P-420 IN VIVO AND THE METHOD OF CALCULATION OF THE ABSORBANCE DUE TO CYTOCHROME P-420

Method of calculation of cytochrome P-420:

A tangent to the slope was drawn and the difference between the absorbance at 420 nm and the tangent was used to obtain values of ΔA.



——— Absorbance spectrum of microsomes from phenobarbital induced rats treated with halothane at time zero and killed 5 hours thereafter.

– – – Absorbance spectrum of microsomes from phenobarbital induced rats killed at a time corresponding to 5 hours after halothane treatment.

TABLE 4.26

THE EFFECTS OF HALOTHANE *IN VIVO* ON THE ACTIVITIES OF HEPATIC HAEM OXYGENASE AND ALA-SYNTHETASE AS A FUNCTION OF TIME IN PHENOBARBITAL INDUCED RATS

All rats were induced with phenobarbital and treated or not with one halothane injection 24 hours later, at time zero. All rats were starved immediately after phenobarbital treatment. Rats were sacrificed at the times after the halothane treatment indicated or at an equivalent time in controls. Results are means ± S.D. for determinations in triplicate on two or more separate groups of three animals.

HOURS AFTER HALOTHANE INJECTION	DOSAGE OF HALOTHANE ml/kg	HAEM OXYGENASE nmoles bilirubin formed /mg mic. protein/10 min.	HAEM OXYGENASE % Relative to controls	ALA-SYNTHETASE µg ALA formed/mg protein/30 min.	ALA-SYNTHETASE % Relative to controls
0	0	0.035 ± 0.003		0.005 ± 0.003	
1	1.0	0.077 ± 0.007*	220	0.013 ± 0.002*	260
5	0	0.045 ± 0.008		0.006 ± 0.002	
5	1.0	0.140 ± 0.014*	331	0.010 ± 0.004	167
10	0	0.077 ± 0.010		0.007 ± 0.001	
10	1.0	0.277 ± 0.024*	360	0.004 ± 0.002*	57

Abbreviations: mic., microsomal; ALA, δ-aminolevulinic acid.

* Differs significantly from identically treated rats not receiving halothane, $P < 0.01$.

hepatic ALA-synthetase was significantly increased relative to controls. However, the activity of ALA-synthetase returned to control levels 5 hours after halothane treatment and was significantly decreased relative to controls at 10 hours (Table 4.26). Furthermore, the activity of ALA-synthetase returned to control levels 24 hours after halothane treatment (Table 4.22).

4.8. CHRONIC HALOTHANE TREATMENT IN VIVO

4.8.1. The effects of chronic halothane treatment on hepatic microsomal enzymes and haem in uninduced rats

These results were obtained from uninduced Wistar rats given repeated halothane injections every 48 hours. The rats were sacrificed 3 hours after the second and sixth injections of halothane. Day 1 - where no halothane had as yet been administered to the rats - was treated as the control.

The levels of hepatic microsomal cytochrome P-450 and haem were significantly decreased after the second and sixth injections of halothane. Furthermore, the extent of loss of hepatic microsomal haem corresponded to the extent of loss of hepatic microsomal cytochrome P-450 (Table 4.27).

The activity of hepatic haem oxygenase was strikingly elevated after the second injection of halothane but decreased to control values after the sixth injection of halothane (Table 4.27).

TABLE 4.27

THE EFFECTS OF CHRONIC HALOTHANE TREATMENT ON HEPATIC MICROSOMAL ENZYMES AND HAEM IN UNINDUCED RATS

Uninduced rats were injected with halothane (1.0 ml/kg) every 48 hours. The rats were sacrificed 3 hours after the second and sixth injections of halothane. Day 1 - where no halothane had as yet been administered to the rats was regarded as the control. Results are reported as means ± S.D. for determinations in triplicate on each of two or more groups of three rats.

DAY	NO. OF HALOTHANE INJECTIONS	CYTOCHROME P-450		HAEM		LOSS HAEM/ LOSS CYTO-CHROME P-450	HAEM OXYGENASE	
		nmoles/mg mic. protein	% Relative to controls	nmoles/mg mic. protein	% Relative to controls	nmoles/mg mic. protein	nmoles bilirubin formed/mg mic. protein/10 min.	% Relative to controls
1	0	1.14 ± 0.06	100	2.04 ± 0.03	100		0.06 ± 0.01	100
5	2	0.68 ± 0.07*	60	1.44 ± 0.11*	71	0.60/0.46	0.19 ± 0.03*	326
13	6	0.73 ± 0.05*	64	1.68 ± 0.05*	82	0.36/0.41	0.07 ± 0.01	122

Abbreviation: mic., microsomal.

* Differs significantly from control rats, $P < 0.01$.

4.8.2. The effects of chronic halothane treatment on the hepatic haem biosynthetic pathway in uninduced rats

4.8.2.1. Urinary haem precursors.

The results of two experiments showing the effects of chronic halothane treatment on the levels of urinary haem precursors are reported in Tables 4.28 - 4.31. Uninduced rats were injected with halothane (1.0 ml/kg) every 48 hours and urine was collected from groups of three identically treated rats over a period of 48 hours, commencing immediately after each halothane injection. Experiments were performed over a period of 22 days (10 injections of halothane). Urine samples were assayed after every second halothane injection. Day 1 - where no halothane had as yet been administered to the rats - was the control for each experiment.

Initially, the levels of urinary ALA and porphobilinogen were significantly increased following 2 and 4 injections of halothane, but returned to control levels after the sixth injection of halothane. After the tenth injection of halothane the levels of ALA and porphobilinogen were again significantly increased relative to controls (Tables 4.28 and 4.29). The levels of urinary uroporphyrin and coproporphyrin were significantly increased relative to controls after the second injection of halothane but subsequently the levels returned to those equivalent to controls or below (Tables 4.30 and 4.31).

4.8.2.2. Hepatic ALA-synthetase

The activity of hepatic ALA-synthetase was assayed in uninduced rats sacrificed 3 hours after the second and sixth injections of halothane. Control animals were those rats to which no halothane had as yet been

TABLE 4.28

THE EFFECTS OF CHRONIC HALOTHANE TREATMENT ON THE LEVELS OF URINARY ALA IN UNINDUCED RATS

Uninduced rats were injected with halothane (1.0 ml/kg) every 48 hours. Urine was collected from groups of three identically treated rats over a period of 48 hours, commencing immediately after each injection of halothane. Urine samples were assayed after every second halothane injection. Day 1 - where no halothane had as yet been administered to the rats - was treated as the control. The results are means for determinations performed in duplicate on each separate group of three rats. For duplicate determinations on one sample, the S.D. value is 1.8.

DAY	NO. OF HALOTHANE INJECTIONS	ALA					
		EXPERIMENT 1		EXPERIMENT 2		AVERAGE	
		µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls
1	0	24.6	100	23.8	100	24.2	100
5	2	68.4	278	101.3	426	84.9^{+}	351
9	4	46.3	188	40.0	168	43.2*	179
13	6	10.9	44	27.9	117	19.4	80
17	8	21.1	86	53.3	224	37.2	154
21	10	110.5	449	60.7	255	85.6*	354

Abbreviation: ALA, δ-aminolevulinic acid. * Differs significantly from control rats, $P < 0.01$.

$^{+}$ Differs significantly from control rats, $P < 0.001$.

TABLE 4.29

THE EFFECTS OF CHRONIC HALOTHANE TREATMENT ON THE LEVELS OF URINARY PORPHOBILINOGEN IN UNINDUCED RATS

Details as in Table 4.28. For duplicate determinations on one sample, the S.D. value is 0.9.

DAY	NO. OF HALOTHANE INJECTIONS	PORPHOBILINOGEN					
		EXPERIMENT 1		EXPERIMENT 2		AVERAGE	
		µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls
1	0	9.4	100	10.2	100	9.8	100
5	2	12.4	132	14.2	139	13.3*	136
9	4	18.0	191	14.4	141	16.2^{+}	165
13	6	4.6	49	11.2	110	7.9	81
17	8	4.7	50	10.1	99	7.4	76
21	10	22.8	243	17.6	173	20.2‡	206

* Probably differs from control rats, $P < 0.05$.

$^{+}$ Differs significantly from control rats, $P < 0.01$.

‡ Differs significantly from control rats, $P < 0.001$.

TABLE 4.30

THE EFFECTS OF CHRONIC HALOTHANE TREATMENT ON THE LEVELS OF URINARY UROPORPHYRIN IN UNINDUCED RATS

Details as in Table 4.28. The results are single determinations performed on each separate group of three rats. For duplicate determinations on one sample, the S.D. value is 0.2.

DAY	NO. OF HALOTHANE INJECTIONS	UROPORPHYRIN					
		EXPERIMENT 1		EXPERIMENT 2		AVERAGE	
		µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls
1	0	0.5	100	0.7	100	0.6	100
5	2	1.9	380	1.8	257	1.9*	317
9	4	0.8	160	0.2	29	0.5	83
13	6	0.4	80	0.2	29	0.3	50
17	8	0.3	60	0.5	71	0.4	67

* Differs significantly from control rats, $P < 0.01$.

TABLE 4.31

THE EFFECTS OF CHRONIC HALOTHANE TREATMENT ON THE LEVELS OF URINARY COPROPORPHYRIN IN UNINDUCED RATS

Details as in Table 4.28. The results are single determinations performed on each separate group of three rats. For duplicate determinations on one sample, the S.D. value is 1.0.

DAY	NO. OF HALOTHANE INJECTIONS	COPROPORPHYRIN					
		EXPERIMENT 1		EXPERIMENT 2		AVERAGE	
		µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls
1	0	5.9	100	6.9	100	6.4	100
5	2	20.0	339	28.8	417	24.4^{+}	381
9	4	5.6	95	1.3	19	3.5	55
13	6	2.7	46	0.8	12	1.8*	28
17	8	2.8	47	4.7	68	3.8	59

* Probably differs from control rats, $P < 0.05$.

$^{+}$ Differs significantly from control rats, $P < 0.01$.

administered (details are given in Table 4.32). The changes in the activity of hepatic ALA-synthetase corresponded to the changes observed in the levels of urinary haem precursors after chronic halothane treatment (cf. Tables 4.28 - 4.31) in that ALA-synthetase activity was significantly increased after the second injection of halothane but was decreased to control values following the sixth injection of halothane (Table 4.32).

4.9. THE EFFECTS OF HALOTHANE IN VITRO

4.9.1. The effects of halothane on the levels of hepatic microsomal cytochrome P-450 and haem in microsomes from phenobarbital induced rats in vitro

The levels of hepatic microsomal cytochrome P-450 and haem were decreased following the incubation of halothane with reaction mixtures containing hepatic microsomes, EDTA and NADPH-generating system (Table 4.33). The losses of hepatic microsomal haem were equivalent to the corresponding losses of microsomal cytochrome P-450 in reaction mixtures incubated with halothane for 0 to 15 min. However, in reaction mixtures incubated with halothane for 25 and 30 min, the losses of hepatic microsomal haem appeared to be less than the corresponding losses of microsomal cytochrome P-450 ($P < 0.05$) (Table 4.33). Incubation of reaction mixtures for 30 min from which either the halothane or the NADPH-generating system was omitted did not result in substantial losses of hepatic microsomal cytochrome P-450 or haem (Table 4.33).

A significant increase in the production of cytochrome P-420 was observed following 30 min incubations of reaction mixtures containing hepatic

TABLE 4.32

THE EFFECTS OF CHRONIC HALOTHANE TREATMENT ON THE ACTIVITY OF HEPATIC ALA-SYNTHETASE IN UNINDUCED RATS

Uninduced rats were injected with halothane (1.0 ml/kg) every 48 hours. The rats were sacrificed 3 hours after the second and sixth injection of halothane. Day 1 - where no halothane had as yet been administered to the rats - was regarded as the control. Results are reported as means ± S.D. for determinations in triplicate on each of two or more groups of three rats.

DAY	NO. OF HALOTHANE INJECTIONS	ALA-SYNTHETASE	
		µg ALA formed/mg protein/30 min	% Relative to controls
1	0	0.003 ± 0.001	100
5	2	0.008 ± 0.002*	267
13	6	0.004 ± 0.001	133

Abbreviation: ALA, δ-aminolevulinic acid.

* Differs significantly from control rats, $P < 0.01$.

TABLE 4.33

THE EFFECTS OF HALOTHANE ON THE LEVELS OF HEPATIC MICROSOMAL CYTOCHROME P-450 AND HAEM IN VITRO

Hepatic microsomes (2 mg protein/ml) from phenobarbital induced Wistar rats, NADPH-generating system and EDTA (0.2 mM) were incubated in the absence or presence of halothane (18 mM) at 30°C with shaking at 60 cycles per min as described in Section 3.3. For the cytochrome P-450 assay, reference cuvettes contained 3 ml of hepatic microsomal suspension (2 mg protein/ml) incubated at 30°C with shaking. Each value corresponds to assays in triplicate or quadruplicate on each of three to four separate preparations of hepatic microsomes, which were prepared from groups of 3 to 5 rats each unless otherwise indicated. Reported losses are relative to zero time samples of identical composition, assayed at 30°. Reported values are means ± S.D. Initial levels in nmoles/mg microsomal protein were: cytochrome P-450, 1.9; haem, 2.8.

ADDITIONS	INCUBATION TIME	CYTOCHROME P-450 LOSS	HAEM LOSS
	min	nmoles/mg mic. protein	nmoles/mg mic. protein
Halothane*	3	0.26 ± 0.08	0.27 ± 0.16
Halothane	5	0.22 ± 0.03	0.24 ± 0.15
Halothane	10	0.31 ± 0.11	0.30 ± 0.13
Halothane	15	0.34 ± 0.10	0.30 ± 0.11
Halothane*	25	0.91 ± 0.09	0.64 ± 0.06‡
Halothane	30	0.81 ± 0.15	0.65 ± 0.36‡§
None	30	0.19 ± 0.09	0.26 ± 0.12
Halothane+	30	0.15 ± 0.08	0.18 ± 0.15

Abbreviation: mic., microsomal.

* Assay on one preparation of hepatic microsomes only.

\+ NADPH-generating system omitted from incubation mixtures.

‡ Probably differs from corresponding loss of cytochrome P-450, $P < 0.05$.

§ Statistical analysis was performed using Student's t-test for paired data.

microsomes, NADPH-generating system, EDTA and halothane relative to 30 min incubations of reaction mixtures from which either the halothane or the NADPH-generating system were omitted (Table 4.34). This is consistent with the difference between the losses of cytochrome P-450 and haem observed in reaction mixtures incubated with halothane for 30 min. However, it was not possible to assess the production of cytochrome P-420 as a function of time, since vortexing halothane into the reaction mixtures at time zero produced measurable levels of cytochrome P-420, and these levels decreased with time in incubation mixtures containing halothane and hepatic microsomes (data not shown).

These results differ quantitatively but not qualitatively from the reports that halothane in part converts cytochrome P-450 to cytochrome P-420 and in part appears to modify the haem moiety of this enzyme in hepatic microsomes *in vitro* (16).

4.10. THE EFFECTS OF HALOTHANE ON DIFFERENT FORMS OF HEPATIC MICROSOMAL CYTOCHROME P-450

Since halothane causes the degradation of hepatic microsomal cytochrome P-450, attempts were made to ascertain which of the multiple forms of cytochrome P-450 were preferentially degraded by halothane *in vitro* and *in vivo* by the use of various enzyme assays. Determination of the rate of ethoxyresofurin deethylation was thought to be characteristic primarily of the major polycyclic hydrocarbon-inducible form of cytochrome P-450 whereas determination of the metyrapone-ferrocytochrome P-450 complex was considered to be characteristic primarily of the major phenobarbital-inducible form of cytochrome P-450 under the conditions of our experiments (260, 265, 282).

TABLE 4.34

THE EFFECT OF HALOTHANE ON THE PRODUCTION OF CYTOCHROME P-420 IN HEPATIC MICROSOMES FROM PHENOBARBITAL TREATED RATS IN VITRO

Experimental details are as given in Table 4.33. Results for each experiment are means for determinations in triplicate on a single preparation of hepatic microsomes prepared from a group of 3 to 5 rats. Individual values were calculated as described in Fig. 4.4.

ADDITIONS	INCUBATION TIME	CYTOCHROME P-420		
		EXPERIMENT 1	EXPERIMENT 2	AVERAGE
	min	ΔA*	ΔA*	ΔA*
None	0	0.035	0.037	0.036±0.001
Halothane	0	0.129	0.096	0.113±0.023
None	30	0.020	0.018	0.019±0.001
Halothane	30	0.108	0.102	0.105±0.014‡
Halothane+	30	0.066	0.058	0.062±0.024

* ΔA = absorbance units, calculated as described in Fig. 4.4.

\+ NADPH-generating system was omitted from incubation mixtures.

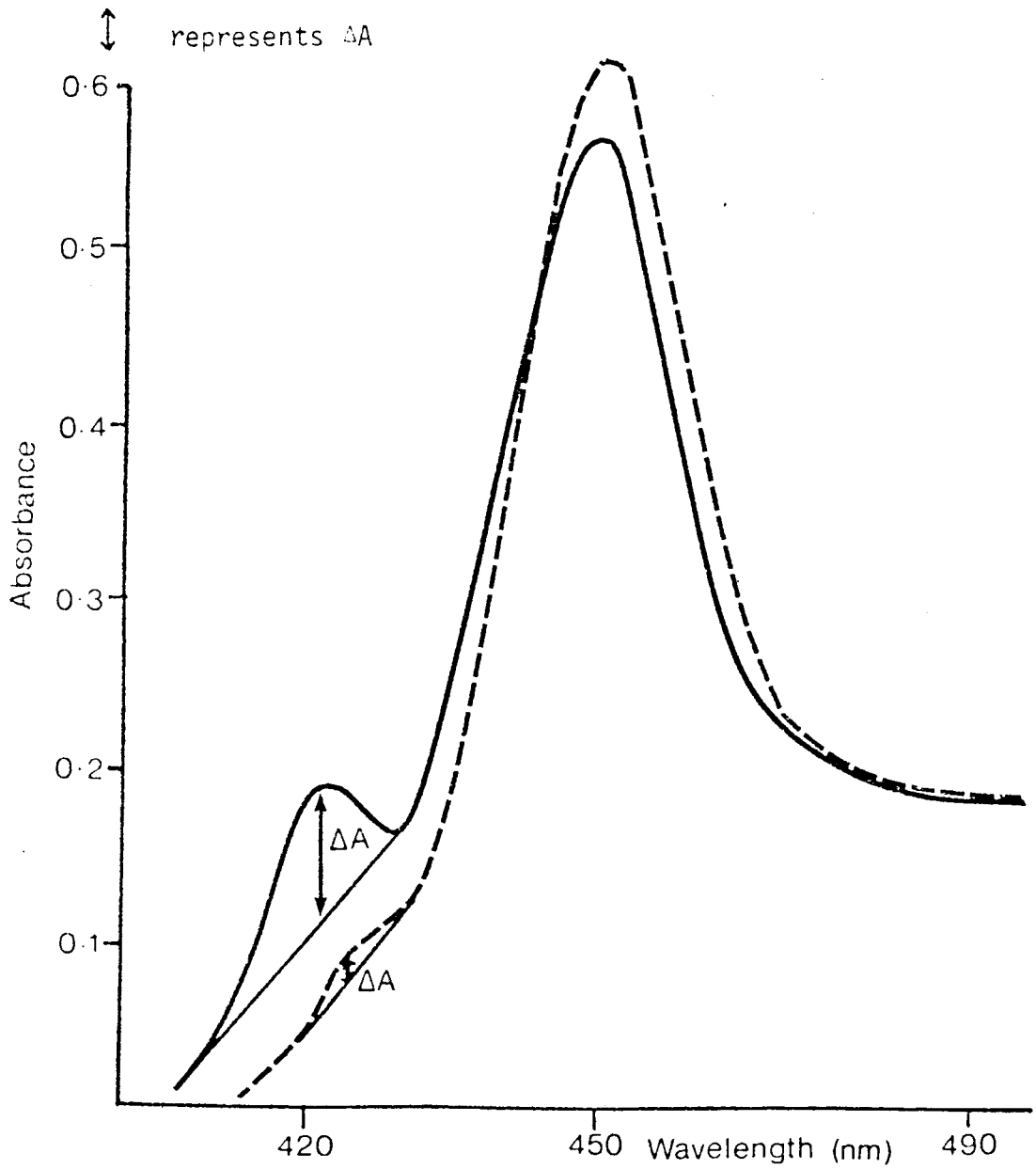
‡ Differs significantly from reaction mixtures from which either halothane or the NADPH-generating system were omitted, $P < 0.01$.

FIGURE 4.4

THE EFFECT OF HALOTHANE ON THE PRODUCTION OF CYTOCHROME P-420 IN VITRO AND THE METHOD OF CALCULATION OF THE ABSORBANCE DUE TO CYTOCHROME P-420

Method of calculation of cytochrome P-420:

A tangent to the slope was drawn and the difference between the absorbance at 420 nm and the tangent was used to obtain values of ΔA.



Absorbance spectrum of hepatic microsomes from phenobarbital induced rats. (——) incubated with halothane, NADPH-generating system and EDTA for 30 min, (- - -) incubated with NADPH-generating system and EDTA for 30 min.

4.10.1. The effects of halothane on the levels and activities of different forms of cytochrome P-450 in microsomes from phenobarbital induced rats in vitro

Following incubation of halothane with hepatic microsomes from phenobarbital induced rats, EDTA and NADPH-generating system, the levels of hepatic microsomal cytochrome P-450 were decreased relative to zero time samples (Table 4.35). At both 15 min and 30 min after halothane treatment in vitro the levels of the major phenobarbital-inducible form of cytochrome P-450 were significantly decreased relative to zero time samples. The losses of the major phenobarbital-inducible form of cytochrome P-450 were equivalent to the losses of cytochrome P-450 (in nmoles per mg of microsomal protein) (Table 4.35). In contrast, halothane caused no significant change in the activity of ethoxyresofurin deethylase in vitro (Table 4.35).

4.10.2. The effects of halothane on the levels and activities of different forms of cytochrome P-450 in phenobarbital induced rats in vivo

Following halothane treatment in vivo, the levels of hepatic microsomal cytochrome P-450 were significantly decreased relative to zero time samples (Table 4.36).

TABLE 4.35

THE EFFECTS OF HALOTHANE ON HEPATIC MICROSOMAL CYTOCHROME P-450 IN VITRO

Details are given in Table 4.33. Metyrapone-ferrocytochrome P-450 levels and ethoxyresofurin deethylase activity were determined as described in Sections 3.5.1.3 and 3.5.1.4. To ensure the validity of the metyrapone-ferrocytochrome P-450 assay, halothane was removed by bubbling with nitrogen for 30 sec. prior to the performance of this assay (282). Each value corresponds to determinations in triplicate or more on one preparation of hepatic microsomes from a group of 5 rats. Determinations at zero time were regarded as the control for each experiment. Reported values are means ± S.D.

TIME min	CYTOCHROME P-450		METYRAPONE-FERROCYTOCHROME P-450	LOSS PB-INDUCIBLE FORM OF CYTOCHROME P-450 / LOSS CYTOCHROME P-450	ETHOXYRESOFURIN DEETHYLASE
	nmoles/mg mic. protein	% Relative to controls	nmoles PB-inducible form of cytochrome P-450/ mg mic. protein	nmoles/mg mic. protein	nmoles ethoxyresofurin deethylated/mg mic. protein
0	1.66 ± 0.10	100	0.80 ± 0.08		0.16 ± 0.03
15	1.37 ± 0.13*	83	0.47 ± 0.06^{+}	0.33 /0.29	0.18 ± 0.04
30	1.09 ± 0.15^{+}	66	0.17 ± 0.03^{+}	0.63 /0.57	0.13 ± 0.01

Abbreviations: mic., microsomal; PB, phenobarbital.

* Probably differs from zero time samples, $P < 0.05$.

$^{+}$ Differs significantly from zero time samples, $P < 0.01$.

TABLE 4.36

THE EFFECTS OF HALOTHANE ON HEPATIC MICROSOMAL CYTOCHROME P-450 IN VIVO

Animals were induced with phenobarbital (80 mg/kg for one day) and treated with one halothane injection (1 ml/kg) 24 hours later at time zero. All rats were starved immediately after phenobarbital treatment and sacrificed at the times after the halothane treatment indicated in the Table. Metyrapone-ferrocytochrome P-450 levels and ethoxyresofurin deethylase activity were determined as described in Sections 3.5.1.3 and 3.5.1.4. Each value corresponds to determinations in triplicate or more on each group of 3 rats. Determinations at zero time were regarded as the control. Reported values are means ± S.D.

TIME min	CYTOCHROME P-450		METYRAPONE-FERROCYTOCHROME P-450	LOSS PB-INDUCIBLE FORM OF CYTOCHROME P-450 / LOSS CYTOCHROME P-450	ETHOXYRESOFURIN DEETHYLASE
	nmoles/mg mic. protein	% Relative to controls	nmoles PB-inducible form of cytochrome P-450/ mg mic. protein	nmoles/mg mic. protein	nmoles ethoxyresofurin deethylated/mg mic. protein
0	1.37 ± 0.01	100	0.88 ± 0.04		0.22 ± 0.01
30	1.12 ± 0.04[+]	82	0.77 ± 0.05[+]	0.11 /0.25	0.14 ± 0.01[+]
60	1.22 ± 0.03[+]	89	0.70 ± 0.04[+]	0.18 /0.15	0.16 ± 0.01[+]

Abbreviation: mic., microsomal; PB, phenobarbital.

[+] Differs significantly from zero time sample, $P < 0.01$.

A significant decrease in the levels of the major phenobarbital-inducible form of cytochrome P-450 was observed 30 and 60 min after halothane treatment *in vivo* (Table 4.36). At the latter time point, viz. 60 min after halothane treatment, the loss of the major phenobarbital-inducible form of cytochrome P-450 was equivalent to the loss of cytochrome P-450 in nmoles per mg of microsomal protein. A small decrease in the activity of ethoxyresofurin deethylase was observed 30 and 60 min after halothane treatment *in vivo* (Table 4.36).

4.11. ACUTE TRICHLOROETHYLENE TREATMENT

4.11.1. The effects of trichloroethylene on hepatic microsomal enzymes and haem in induced and uninduced rats

4.11.1.1. Cytochrome P-450 and haem.

The effects of acute trichloroethylene treatment on the levels of hepatic microsomal cytochrome P-450 and haem were determined in phenobarbital induced and uninduced Wistar rats (Table 4.37). Significant decreases in the levels of cytochrome P-450 and haem were observed in both phenobarbital induced and uninduced rats 24 hours after trichloroethylene treatment. The losses of cytochrome P-450 and haem in nmoles/mg of microsomal protein were greater in phenobarbital induced than in uninduced rats, although the percentage loss of cytochrome P-450 was greater in uninduced than in phenobarbital induced rats. The losses of haem were similar to the losses of cytochrome P-450 in all experiments ($P > 0.05$) (Table 4.37).

TABLE 4.37

THE EFFECTS OF TRICHLOROETHYLENE ON HEPATIC MICROSOMAL ENZYMES AND HAEM IN PHENOBARBITAL INDUCED AND UNINDUCED RATS

Rats were sacrificed 24 hours after trichloroethylene treatment or at an equivalent time in control animals. Results are means ± S.D. for assays performed in triplicate on each of two or more separate groups of three animals.

INDUCTION	DOSAGE OF TCE ml/kg	CYTOCHROME P-450 nmoles/mg mic. protein	% Relative to controls	HAEM nmoles/mg mic. protein	% Relative to controls	LOSS HAEM/ LOSS CYTO-CHROME P-450 nmoles/mg mic. protein	HAEM OXYGENASE nmoles bilirubin formed/mg mic. protein	% Relative to controls
NONE	None	1.05 ± 0.13		2.04 ± 0.24			0.110 ± 0.018	
	2.0	0.71 ± 0.08*	68	1.51 ± 0.18*	69	0.53/0.40	0.419 ± 0.074*	381
PB	None	1.84 ± 0.04		2.67 ± 0.16			0.104 + 0.014	
	1.0	1.09 ± 0.05*	59	2.05 ± 0.08*	77	0.62/0.75	0.237 ± 0.037*	228

Abbreviations: mic., microsomal; PB, phenobarbital; TCE, trichloroethylene.

* Differs significantly from value for identically induced rats not receiving trichloroethylene, $P < 0.01$.

4.11.1.2. Haem oxygenase.

Twenty-four hours after acute trichloroethylene treatment, significant increases in the activity of hepatic haem oxygenase were observed in both phenobarbital induced and uninduced rats. However, a greater increase in haem oxygenase activity was observed in uninduced rats than in phenobarbital induced rats (Table 4.37).

4.11.2. The effects of trichloroethylene on the hepatic haem biosynthetic pathway in induced and uninduced rats

The effects of acute trichloroethylene treatment on the levels of urinary precursors in induced and uninduced rats are shown in Table 4.38. No significant changes in the levels of urinary haem precursors were observed in uninduced rats after acute trichloroethylene treatment. A significant increase in the levels of porphobilinogen was observed in the urine of phenobarbital induced rats after acute trichloroethylene treatment, but the levels of the other urinary haem precursors measured, viz. ALA, uroporphyrin and coproporphyrin were unchanged relative to control animals (Table 4.38).

No significant changes in the activity of ALA-synthetase were observed in either phenobarbital induced or uninduced rats, 24 hours after acute trichloroethylene treatment. The percentages of the enzyme activity after trichloroethylene treatment relative to its activity in identically induced rats not receiving trichloroethylene were as follows:- uninduced rats, 120%; phenobarbital induced rats, 100%.

TABLE 4.38

THE EFFECTS OF TRICHLOROETHYLENE ON THE LEVELS OF URINARY HAEM PRECURSORS IN PHENOBARBITAL INDUCED AND UNINDUCED RATS

Urine was collected from groups of three identically treated rats over a period of 24 hours, commencing immediately after the trichloroethylene injection or at an equivalent time in control rats. Results are means ± S.D. for determinations on each of two or more groups of three rats. S.D. values for duplicate determinations on one sample are given in Tables 4.39 - 4.42.

INDUCTION	DOSAGE OF TCE ml/kg	ALA µg/24 hrs /3 rats	ALA % Relative to controls	PORPHOBILINOGEN µg/24 hrs /3 rats	PORPHOBILINOGEN % Relative to controls	UROPORPHYRIN µg/24 hrs /3 rats	UROPORPHYRIN % Relative to controls	COPROPORPHYRIN µg/24 hrs /3 rats	COPROPORPHYRIN % Relative to controls
NONE	None	84 ± 5		12 ± 3		1.8 ± 0.9		32.4 ± 17.3	
	2.0	73 ± 6	87	16 ± 7	133	1.2 ± 0.3	67	14.1 ± 6.6	44
PB	None	110 ± 13		15 ± 1		0.7 ± 0.1		41.6 ± 15.6	
	1.0	115 ± 15	105	23 ± 2*	153	0.8 ± 0.5	114	42.8 ± 13.0	103

Abbreviations: TCE, trichloroethylene; PB, phenobarbital; ALA, δ-aminolevulinic acid.

* Differs significantly from identically induced rats not treated with trichloroethylene, $P < 0.01$.

4.12. CHRONIC TRICHLOROETHYLENE TREATMENT

4.12.1. The effects of chronic trichloroethylene treatment on the levels of haem precursors in uninduced rats

Uninduced rats were injected with trichloroethylene (1.0 ml/kg) every 48 hours. Urine and faeces were collected from two groups of three identically treated rats over a period of 48 hours, commencing immediately after each trichloroethylene injection. Day 1 - where no trichloroethylene had as yet been administered to the rats - was regarded as the control for each experiment.

The results of two experiments showing the effects of chronic trichloroethylene treatment on the levels of urinary and faecal haem precursors over a period of 19 days (or 8 injections of trichloroethylene) are reported in Tables 4.39 - 4.44. The levels of urinary ALA were probably significantly increased relative to control values after the first and second injections of trichloroethylene but subsequently decreased until the levels were unchanged relative to control levels or below (Table 4.39). Urinary porphobilinogen levels were increased relative to control values ($P < 0.05$) after the first injection of trichloroethylene but in general porphobilinogen levels remained unchanged relative to control levels for the remainder of the experiments ($P > 0.1$) (Table 4.40). However, uroporphyrin levels were strikingly elevated after the first injection of trichloroethylene in both experiments and remained elevated until the sixth injection of trichloroethylene, after which the levels of uroporphyrin decreased to control levels (Table 4.41). Urinary coporporphyrin levels were also strikingly elevated after the first and second injections of trichloroethylene but the levels gradually decreased until they were equivalent to control levels (Table 4.42).

TABLE 4.39

THE EFFECTS OF CHRONIC TRICHLOROETHYLENE TREATMENT ON THE LEVELS OF URINARY ALA IN UNINDUCED RATS

Uninduced rats were injected with trichloroethylene (1.0 ml/kg) every 48 hours. Urine was collected from groups of three identically treated rats over a period of 48 hours, commencing immediately after each injection of trichloroethylene. Day 1 - where no trichloroethylene had as yet been administered to the rats - was treated as the control. The results are means for determinations performed in duplicate on each separate group of three rats. For duplicate determinations on one sample, the S.D. value is 1.8.

DAY	NO. OF TRICHLOROETHYLENE INJECTIONS	ALA					
		EXPERIMENT 1		EXPERIMENT 2		AVERAGE	
		µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls
1	0	62.0	100	19.7	100	40.9	100
3	1	79.6	128	130.7	663	105.2*	257
5	2	90.2	145	60.0	305	75.1*	184
7	3	81.8	132	35.4	180	58.6	143
9	4	86.7	140	13.1	66	49.9	122
11	5	78.3	126	12.5	63	45.4	111
13	6	42.7	69	40.4	205	41.6	102
15	7	21.1	34	-	-	-	-
17	8	19.4	31	19.3	98	19.4	47

Abbreviation: ALA, δ-aminolevulinic acid.

* Probably differs from control rats, $P < 0.05$.

TABLE 4.40

THE EFFECTS OF CHRONIC TRICHLOROETHYLENE TREATMENT ON THE LEVELS OF URINARY PORPHOBILINOGEN IN UNINDUCED RATS

Details as in Table 4.39. For duplicate determinations on one sample, the S.D. value is 0.9.

DAY	NO. OF TRICHLOROETHYLENE INJECTIONS	PORPHOBILINOGEN					
		EXPERIMENT 1		EXPERIMENT 2		AVERAGE	
		µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls
1	0	20.0	100	5.1	100	12.6	100
3	1	23.6	118	30.3	594	27.0*	214
5	2	18.9	95	17.3	339	18.1	144
7	3	10.6	53	6.2	122	8.4	67
9	4	16.2	81	7.4	145	11.8	94
11	5	11.6	58	2.0	39	6.8	54
13	6	15.9	80	12.2	239	14.1	112
15	7	9.7	49	14.7	288	12.2	97
17	8	7.4	37	2.5	49	5.0	40

* Probably differs from control rats, $P < 0.05$.

TABLE 4.41

THE EFFECTS OF CHRONIC TRICHLOROETHYLENE TREATMENT ON THE LEVELS OF URINARY UROPORPHYRIN IN UNINDUCED RATS

Details as in Table 4.39. The results are single determinations performed on a group of three rats. For duplicate determinations on one sample, the S.D. value is 0.1.

DAY	NO. OF TRICHLOROETHYLENE INJECTIONS	UROPORPHYRIN					
		EXPERIMENT 1		EXPERIMENT 2		AVERAGE	
		µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls
1	0	0.5	100	0.1	100	0.3	100
3	1	2.9	580	1.8	1080	2.4	800
5	2	2.2	440	0.4	400	1.3	433
7	3	1.3	260	0.3	300	0.8	267
9	4	-	-	0.3	300	-	-
11	5	1.6	320	0.1	100	0.9	300
13	6	0.5	100	0.7	700	0.6	200
15	7	0.3	60	0.5	500	0.4	133
17	8	0.4	80	0.3	300	0.4	133

TABLE 4.42

THE EFFECTS OF CHRONIC TRICHLOROETHYLENE TREATMENT ON THE LEVELS OF URINARY COPROPORPHYRIN IN UNINDUCED RATS

Details as in Table 4.39. The results are single determinations performed on a group of three rats. For duplicate determinations on one sample, the S.D. value is 1.0.

DAY	NO. OF TRICHLOROETHYLENE INJECTIONS	COPROPORPHYRIN					
		EXPERIMENT 1		EXPERIMENT 2		AVERAGE	
		µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls	µg/48 hrs /3 rats	% Relative to controls
1	0	5.9	100	1.4	100	3.7	100
3	1	24.7	419	16.5	1178	20.6	557
5	2	34.5	585	7.9	564	21.2	573
7	3	12.8	217	2.5	179	7.7	208
9	4	10.3	175	3.8	271	7.1	192
11	5	22.2	376	1.4	100	11.8	319
13	6	4.6	78	5.2	371	4.9	132
15	7	4.4	75	3.1	221	3.8	103
17	8	7.3	124	1.4	100	4.4	119

Faecal coporporphyrin and protoporphyrin levels were elevated after the second injection of trichloroethylene relative to control values but the levels remained unchanged throughout the rest of the experiments (Tables 4.43, 4.44).

4.13. ACUTE TETRACHLOROETHYLENE TREATMENT

4.13.1. The effects of tetrachloroethylene on hepatic microsomal enzymes and haem in phenobarbital induced rats

4.13.1.1. Cytochrome P-450 and haem.

The effects of acute tetrachloroethylene on the levels of hepatic microsomal cytochrome P-450 and haem were assessed in phenobarbital induced Wistar rats (Table 4.45). A single injection of tetrachloroethylene into phenobarbital induced rats resulted in a significant decrease in the levels of hepatic microsomal cytochrome P-450, but not of microsomal haem (Table 4.45).

4.13.1.2. Haem oxygenase.

A slight increase in the activity of hepatic haem oxygenase was observed after acute tetrachloroethylene treatment of phenobarbital induced rats (Table 4.46).

4.13.2. The effects of tetrachloroethylene on hepatic haem biosynthesis in phenobarbital induced rats

Acute tetrachloroethylene treatment of phenobarbital induced rats caused no significant alteration in the levels of the urinary haem precursors (Table 4.47) or in the activity of hepatic ALA-synthetase (Table 4.46).

TABLE 4.43

THE EFFECTS OF CHRONIC TRICHLOROETHYLENE TREATMENT ON THE LEVELS OF FAECAL COPROPORPHYRIN IN UNINDUCED RATS

Uninduced rats were injected with trichloroethylene (1.0 ml/kg) every 48 hours. Faeces was collected from groups of three identically treated rats over a period of 48 hours, commencing immediately after each injection of trichloroethylene. Day 1 - where no trichloroethylene had as yet been administered to the rats - was treated as the control. The results are single determinations performed on each separate group of three rats. For duplicate determinations on one sample, the S.D. value is 4.0.

DAY	NO. OF TRICHLOROETHYLENE INJECTIONS	COPROPORPHYRIN					
		EXPERIMENT 1		EXPERIMENT 2		AVERAGE	
		µg/g dry wt. /3 rats	% Relative to controls	µg/g dry wt. /3 rats	% Relative to controls	µg/g dry wt. /3 rats	% Relative to controls
1	0	7.8	100	8.7	100	8.3	100
3	1	9.6	123	3.8	44	6.7	81
5	2	16.4	210	11.8	136	14.1	170
7	3	13.8	177	4.8	55	9.3	112
9	4	10.7	137	8.0	92	9.4	113
11	5	9.2	118	7.2	83	8.2	99
13	6	10.8	138	5.0	57	7.9	95
15	7	8.6	110	6.7	77	7.7	93
17	8	5.6	72	6.7	77	6.2	75

Abbreviation: wt., weight.

TABLE 4.44

THE EFFECTS OF CHRONIC TRICHLOROETHYLENE TREATMENT ON THE LEVELS OF FAECAL PROTOPORPHYRIN IN UNINDUCED RATS

Details as in Table 4.43. For duplicate determinations on one sample, the S.D. value is 8.5.

DAY	NO. OF TRICHLOROETHYLENE INJECTIONS	PROTOPORPHYRIN					
		EXPERIMENT 1		EXPERIMENT 2		AVERAGE	
		µg/g dry wt. /3 rats	% Relative to controls	µg/g dry wt. /3 rats	% Relative to controls	µg/g dry wt. /3 rats	% Relative to controls
1	0	59.8	100	47.8	100	53.8	100
3	1	41.4	69	62.4	131	51.9	96
5	2	102.5	171	86.6	181	94.6	176
7	3	89.4	149	64.3	135	76.9	143
9	4	43.9	73	-	-	-	-
11	5	38.3	64	49.5	104	43.9	82
13	6	66.1	111	27.2	57	46.7	87
15	7	38.4	64	45.4	95	41.9	78
17	8	45.3	76	35.1	73	40.2	75

Abbreviation: wt., weight.

TABLE 4.45

THE EFFECTS OF TETRACHLOROETHYLENE ON THE LEVELS OF HEPATIC MICROSOMAL CYTOCHROME P-450 AND HAEM IN PHENOBARBITAL INDUCED RATS

Rats were sacrificed 24 hours after tetrachloroethylene treatment or at an equivalent time in control animals. Results are means ± S.D. for assays performed in triplicate on each of two separate groups of three animals.

TETRACHLOROETHYLENE	CYTOCHROME P-450		HAEM		LOSS HAEM/LOSS CYTOCHROME P-450
ml/kg	nmoles/mg mic. protein	% Relative to controls	nmoles/mg mic. protein	% Relative to controls	nmoles/mg mic. protein
None	2.08 ± 0.04		2.86 ± 0.11		
1.0	1.57 ± 0.10*	75	2.68 ± 0.05	94	0.18/0.51+

Abbreviations: mic., microsomal.

* Differs significantly from identically induced animals not treated with tetrachloroethylene, $P < 0.01$.

+ Loss of cytochrome P-450 differs significantly from loss of haem (in nmoles/mg mic. protein), $P < 0.01$.

TABLE 4.46

THE EFFECTS OF TETRACHLOROETHYLENE ON THE ACTIVITIES OF HEPATIC HAEM OXYGENASE AND ALA-SYNTHETASE IN PHENOBARBITAL INDUCED RATS

Rats were sacrificed 24 hours after tetrachloroethylene treatment or at an equivalent time in control animals.

Results are means ± S.D. for assays performed in triplicate or more on each of two separate groups of three rats.

TETRACHLOROETHYLENE	HAEM OXYGENASE		ALA-SYNTHETASE	
ml/kg	nmoles bilirubin formed /mg mic. protein/10 min	% Relative to controls	µg ALA formed /mg protein/30 min	% Relative to controls
None	0.084 ± 0.017		0.004 ± 0.001	
1.0	0.160 ± 0.072*	190	0.005 ± 0.001	125

Abbreviations: mic., microsomal; ALA, δ-aminolevulinic acid.

* Probably differs from identically induced rats not treated with tetrachloroethylene, $P < 0.05$.

TABLE 4.47

THE EFFECTS OF TETRACHLOROETHYLENE ON THE LEVELS OF URINARY HAEM PRECURSORS IN PHENOBARBITAL INDUCED RATS

Results are from a single experiment only. Urine was collected from groups of three identically treated rats over a period of 24 hours, commencing immediately after tetrachloroehtylene treatment or at an equivalent time in controls rats. For determinations of the levels of ALA and porphobilinogen, results are means for duplicate determinations on each group of three rats whereas for determinations of the levels of uroporphyrin and coproporphyrin results are single determinations only. S.D. values for duplicate determinations on one sample are given in Tables 4.39 - 4.42.

DOSAGE OF TETRACHLOROETHYLENE ml/kg	ALA		PORPHOBILINOGEN		UROPORPHYRIN		COPROPORPHYRIN	
	µg/24 hrs /3 rats	% Relative to controls	µg/24 hrs /3 rats	% Relative to controls	µg/24 hrs /3 rats	% Relative to controls	µg/24 hrs /3 rats	% Relative to controls
None	62		14		1.3		23.1	
1.0	85	137	9	64	1.3	100	29.5	128

V DISCUSSION

Results presented in this thesis demonstrate that intraperitoneal administration of the anaesthetic agents, fluroxene, halothane and trichloroethylene, causes a decrease in the levels of hepatic microsomal cytochrome P-450 *in vivo* and also affects the rates of hepatic haem biosynthesis and biodegradation (Sections 4.1 - 4.13). Several aspects of these findings will be discussed in detail, namely:

(1) The diverse ways in which these compounds decrease the levels of cytochrome P-450;

(2) The role of the extent and the type of destruction of cytochrome P-450 in causing alterations in the rates of hepatic haem biosynthesis and biodegradation; and

(3) Other mechanisms whereby these and other chemicals might affect the rates of haem metabolism in the liver.

In view of their effects on haem biosynthesis in the rat, the three anaesthetic agents will be assessed as providing possible experimental models for human genetic porphyria.

As mentioned in the Introduction (Section 1.6), the levels of hepatic microsomal cytochrome P-450 have been postulated by several workers to be an important factor in determining the rates of hepatic haem biosynthesis and biodegradation (133, 215, 283, 284). The effects of cytochrome P-450 on hepatic haem metabolism are thought to arise from (a) the ability of the apoprotein of cytochrome P-450 to draw haem from a central hepatic pool of haem which regulates the rates of hepatic haem

biosynthesis and biodegradation (Section 1.6) and (b) the ability of cytochrome P-450 to dissociate into haem and apoprotein, with the haem entering the central haem pool (Section 1.6).

It has been found that several xenobiotics which alter the levels of hepatic cytochrome P-450, perturb haem biosynthesis (215, 285) and/or haem biodegradation (133, 284) (cf. Sections 1.8.1.3 and 1.9). For example, the chemical AIA, which degrades the haem moiety of hepatic microsomal cytochrome P-450 (Section 1.8.1.3) (121, 245, 246) is commonly used to induce a state of experimental porphyria in laboratory animals.

5.1. FLUROXENE

At the onset of this project, the degradation of hepatic microsomal cytochrome P-450 by the anaesthetic agent fluroxene (Section 1.3.1) appeared to be similar to that mediated by AIA (Section 1.8.1.3) in that both chemicals specifically degraded the haem moiety of the enzyme and the reaction kinetics of the degradation reaction were, in both cases, pseudo first order (136). Thus it was decided to determine the effects of fluroxene on hepatic haem metabolism *in vivo* in order to test the proposal that an alteration in the levels of cytochrome P-450 can affect haem metabolism (Section 1.6) (133, 215, 283, 284).

In our initial experiments, it was confirmed that intraperitoneal administration of fluroxene to Wistar rats resulted in the degradation of the haem moiety of hepatic microsomal cytochrome P-450, i.e. the loss of microsomal haem was equivalent to the loss of cytochrome P-450 (in nmoles per mg of microsomal protein) after acute fluroxene treatment

(Table 4.1)[+].

Acute fluroxene treatment also resulted in increases in the rates of both hepatic haem biosynthesis and biodegradation (Tables 4.2 - 4.7). The effects of fluroxene on hepatic haem metabolism appeared to be related to the fluroxene mediated degradation of cytochrome P-450, since TFEE (Fig. 1.5), the saturated analogue of fluroxene, which does not cause degradation of hepatic cytochrome P-450 (Table 4.8) (65, 72, 135) did not affect hepatic haem biosynthesis or biodegradation (Tables 4.8, 4.9). Furthermore, in general, pretreatment of the experimental animals with phenobarbital or 3-methylcholanthrene enhanced both the fluroxene mediated degradation of cytochrome P-450 and the effects of fluroxene on the pathways of haem biosynthesis and biodegradation (Tables 4.1 - 4.7).

The specifics of the effects of fluroxene on hepatic haem metabolism are discussed below:

Firstly, acute fluroxene treatment resulted in an increase in the activity of the rate-limiting enzyme of haem biosynthesis, ALA-synthetase (Fig. 1.11) (Table 4.7). Hepatic ALA-synthetase has been

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[+] Although fluroxene contains 0.01% N-phenyl-1-naphthylamine, an antioxidant which may be capable of stimulating lipid peroxidation, the effects of fluroxene on hepatic microsomal cytochrome P-450 do not appear to arise from lipid peroxidation since experiments with fluroxene *in vitro* were conducted in the presence of EDTA, which is an inhibitor of lipid peroxidation. In addition, pronounced degradation of cytochrome P-450 by fluroxene occurred under conditions where lipid peroxidation was negligible (as assessed by malonaldehyde production) (65).

found to be induced by a variety of hydrophobic compounds (286,287). Induction of hepatic ALA-synthetase has been attributed to a derepression of the synthesis of the enzyme, caused by a loss of haem from the regulatory haem pool which decreases the feedback inhibition of haem on ALA-synthetase (215, 220, 286). Therefore, the induction of ALA-synthetase observed in fluroxene treated rats (Table 4.7) is presumably due to a loss of haem from the hepatic haem pool which regulates the activity of ALA-synthetase).

There appear to be two possible mechanisms by which fluroxene could deplete the hepatic haem pool. Firstly, large amounts of hepatic haem are presumably required to replace the haem of hepatic cytochrome P-450 degraded by fluroxene (Table 4.1). Secondly, acute fluroxene treatment resulted in an increase in the rate of hepatic haem biodegradation (Tables 4.2, 4.3) which could further deplete hepatic haem levels in the regulatory haem pool and lead to the induction of ALA-synthetase (Table 4.7).

The fluroxene mediated induction of hepatic ALA-synthetase (Table 4.7) resulted in an increase in the rate of hepatic haem biosynthesis in fluroxene treated animals, i.e. increases in the levels of the urinary haem precursors ALA and porphobilinogen were observed after acute fluroxene treatment (Table 4.4). However, in general, no significant increases in the levels of the urinary and faecal porphyrins were observed after fluroxene treatment (Tables 4.5, 4.6). This would appear to arise from a situation where uroporphyrinogen synthetase (Fig. 1.11) is the rate-limiting enzyme of the haem biosynthetic pathway. This enzyme normally functions at a marginally greater rate than ALA-synthetase, which is, under normal circumstances, the rate-determining

enzyme of haem biosynthesis (153, 154). Uroporphyrinogen synthetase may therefore simply become the rate-limiting step in haem biosynthesis because the activity of ALA-synthetase is elevated (288). Alternatively, it is possible that acute fluroxene treatment may have decreased the activity of this enzyme, as was found after the chronic exposure of animals to fluroxene (Section 4.3).

As mentioned earlier, acute fluroxene treatment was also found to cause a pronounced elevation in the rate of hepatic haem biodegradation, i.e. an increase in the activity of hepatic haem oxygenase was observed after treatment with fluroxene (Table 4.2). The elevation of this enzyme in fluroxene treated rats appeared to be linked to the fluroxene mediated degradation of cytochrome P-450 since, following fluroxene treatment of differently induced animals, there was a direct relationship between the loss of the haem of hepatic microsomal cytochrome P-450 and the increase in hepatic haem oxygenase activity (Table 4.2). Furthermore, the degradation of cytochrome P-450 occurred prior to the elevation of hepatic haem oxygenase in fluroxene treated rats (Table 4.3), which is consistent with the suggestion that the induction of hepatic haem oxygenase may be a consequence of the fluroxene mediated degradation of cytochrome P-450.

In contrast to the effects of fluroxene on hepatic haem oxygenase activity, AIA, which also degrades the haem moiety of hepatic microsomal cytochrome P-450 (Section 1.8.1.3) does not affect the activity of hepatic haem oxygenase in vivo (248). Thus the degradation of the haem moiety of hepatic cytochrome P-450 does not in itself appear to be the only prerequisite for the induction of hepatic haem oxygenase in fluroxene treated animals. It is possible that the adduct of

fluroxene with the haem of cytochrome P-450 (which differs in structure from the AIA-haem adduct (Sections 1.3.1, 1.8.1.3)) induces the activity of hepatic haem oxygenase. Alternatively, it is possible that the fluroxene mediated increase in hepatic haem oxygenase activity arises from elevated levels of ferric ions, which were perhaps released from the fluroxene-haem adduct. The de novo induction of hepatic haem oxygenase by haem as well as by certain heavy metals, including iron, has been well documented (289-294).

The concurrent increases in the activities of hepatic ALA-synthetase and hepatic haem oxygenase which were observed after acute fluroxene treatment (Tables 4.2, 4.7) were unexpected, since an increase in hepatic ALA-synthetase activity is associated with a loss of haem from the regulatory haem pool (215, 220), whereas an increase in haem oxygenase activity is generally thought to be a consequence of an increase in the level of haem in the regulatory pool (295) (Section 1.6). In order to determine more precisely the mechanisms by which acute fluroxene treatment affects hepatic haem metabolism, experiments should be performed in which the levels of ALA-synthetase, haem oxygenase and cytochrome P-450 are determined as a function of time after fluroxene administration (similar to the experiment described in Table 4.3). Changes in the levels of haem in the regulatory haem pool should also be monitored at various time intervals after fluroxene treatment - an experiment which is just becoming feasible via sensitive techniques such as chemiluminescence (296). Furthermore, the effects of the adduct of fluroxene with the haem of cytochrome P-450 on hepatic haem oxygenase should be assessed.

With a view to determining the effects of the fluroxene-haem adduct on hepatic haem oxygenase activity, we attempted to isolate and characterize this adduct in our laboratory (Sections 3.8, 4.5). The results of our experiments indicated that the haem adduct was an N-substituted porphyrin (Section 4.5) (Fig. 4.1). This was later confirmed by Kunze et al. (138) who identified the haem adduct as N-(2-oxo-ethyl)-protoporphyrin IX. The identical N-substituted porphyrin has also been identified in the livers of rats treated with vinyl fluoride, vinyl bromide and acetylene (297). The formation of the same haem adduct by all four substrates indicates that an oxygen is introduced at the trifluoroethoxy or halide substituted terminus of the π bond, followed by reaction of the unsubstituted terminus with the nitrogen atom of the haem of cytochrome P-450 (297). The most recent investigations into the formation of N-(2-oxo-ethyl)-protoporphyrin IX suggest that the fluroxene mediated alkylation of cytochrome P-450 prosthetic haem occurs via a radical intermediate (297) and not via a cationic intermediate as previously suggested (69). However, the precise mechanism of formation of the haem adduct remains to be elucidated.

Once the fluroxene-haem adduct had been isolated and characterized in our laboratory, it was attempted to assess the effects of this compound on hepatic haem oxygenase activity in vitro. Under the conditions of our experiments, the isolated adduct did not affect hepatic haem oxygenase activity (results not shown). However, these results should be viewed with circumspection due to the instability of the isolated adduct[+] and the observation that the isolation procedure used resulted

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[+] The isolated adduct, N-(2-oxo-ethyl)-protoporphyrin IX, is highly unstable and decomposes overnight in liquid nitrogen (-195°C) (results not shown) (281, 297).

in the loss of the iron ion, which is thought to be an integral part of the haem adduct in vivo (130, 297). Consequently, the effects of the isolated adduct on hepatic haem oxygenase activity in vitro might differ from the effects of the adduct on this enzyme in vivo.

In contrast to acute fluroxene treatment, chronic fluroxene treatment of laboratory animals did not appear to affect the levels of hepatic microsomal cytochrome P-450 and the activity of hepatic haem oxygenase (Table 4.10). Some effect of chronic fluroxene administration on these enzymes might have been evident if their activities had been assessed as a function of time. However, it is not unusual to observe different consequences of chronic versus acute administration of a drug (298).

Chronic fluroxene treatment resulted in an increased rate of hepatic haem biosynthesis (Tables 4.11 - 4.17) which was far more striking than that observed after acute fluroxene administration (Tables 4.4 - 4.7). The induction of hepatic haem biosynthesis after chronic fluroxene treatment appears to arise from a different mechanism than that proposed for acute fluroxene administration. While the ability of acute fluroxene treatment to induce hepatic haem biosynthesis appears to be a consequence of the fluroxene mediated degradation of hepatic microsomal cytochrome P-450 (Table 4.1), it is proposed that the ability of chronic fluroxene treatment to stimulate hepatic haem biosynthesis is a result of the inhibition of hepatic uroporphyrinogen synthetase in these animals (Table 4.17).

Prolonged administration of fluroxene to uninduced animals produced a pronounced decrease (approximately 50%) in the activity of hepatic

uroporphyrinogen synthetase (Table 4.17). The decreased activity of hepatic uroporphyrinogen synthetase after chronic fluroxene treatment would cause a partial block in hepatic haem biosynthesis and would thereby decrease the repression of ALA-synthetase by haem, thus explaining the observed elevation in the activity of the latter enzyme (Table 4.17) (299, 300). Consistent with the above effects, increased levels of porphobilinogen and to a lesser extent, ALA, were observed in the urine of animals after chronic fluroxene treatment (Tables 4.11, 4.12) (266).

The mechanism responsible for the decrease in hepatic uroporphyrinogen synthetase activity observed after chronic fluroxene treatment of animals, is not known. It does not appear to be related to excessive ALA-synthetase activity, since the activity of uroporphyrinogen synthetase is not diminished by induction of ALA-synthetase in rats treated with AIA (301). In addition, high concentrations of porphobilinogen do not appear to inhibit uroporphyrinogen synthetase activity (299).

A possible explanation for the observed decrease in the activity of uroporphyrinogen synthetase after chronic fluroxene treatment is that fluroxene, or a metabolite thereof, is directly affecting the activity or levels of this enzyme. However, neither fluroxene nor its metabolites 2,2,2-trifluoroethanol (TFE), trifluoroacetic acid and trifluoroacetaldehyde had any effect on the activity of hepatic uroporphyrinogen synthetase *in vitro* (Table 4.19). It appeared possible therefore, that the haem adduct produced by fluroxene treatment could inhibit hepatic uroporphyrinogen synthetase. The ability of the haem adduct produced by DDC treatment of animals, namely N-methylprotoporphyrin IX, to inhibit the enzyme of the haem biosynthetic pathway, ferrochelatase, is well

documented (237-239). Thus attempts were made to assess the effects of the isolated fluroxene-haem adduct on hepatic uroporphyrinogen synthetase in vitro (Table 4.20). Although, under the conditions of our experiments, the fluroxene-haem adduct caused no change in the activity of hepatic uroporphyrinogen synthetase (Table 4.20), these results are not conclusive in view of [1] the instability of the isolated adduct (281, 297), [2] the presence of high concentrations of acid in the isolated product which required neutralization, a process which resulted in the partial destruction of the adduct (results not shown), and [3] the probable loss of the iron ion of the adduct due to the isolation procedures used (see above) (297). Thus further experimentation is required in order to assess the proposal that the haem adduct is associated with the inhibition of hepatic uroporphyrinogen synthetase following chronic fluroxene treatment. Furthermore, since the same haem adduct is produced by not only fluroxene, but also vinyl fluoride, vinyl bromide and acetylene, the effects of these compounds on hepatic haem biosynthesis and in particular, hepatic uroporphyrinogen synthetase could be determined in vivo. If the haem adduct is indeed responsible for the inhibition of hepatic uroporphyrinogen synthetase after chronic fluroxene treatment, chronic administration of vinyl fluoride, vinyl bromide and acetylene to experimental animals would be expected to produce similar results to those observed after chronic fluroxene treatment, viz. inhibition of hepatic uroporphyrinogen synthetase, induction of hepatic ALA-synthetase and increased levels of porphobilinogen and to a lesser extent, ALA.

The effects of chronic fluroxene administration on the hepatic haem biosynthetic pathway, in particular the inhibition of uroporphyrinogen synthetase, are extremely relevant to the study of the human genetic diseases

known as the porphyrias (Section 1.7). One of the several types of acute porphyrias, namely Acute Intermittent Porphyria (AIP) is characterized biochemically by a defect in the activity of hepatic uroporphyrinogen synthetase. In AIP the activity of this enzyme is reduced to approximately 50% of normal levels (302-304). This enzyme defect is transmitted as an autosomal dominant and manifests itself in all tissues, including the liver (302).

The precise nature of the inherited defect in uroporphyrinogen synthetase is now known. Anderson and Desnick (J. Clin. Invest. 68, 1 (1981)) have purified the enzyme and provided a detailed scheme for its mechanism and alteration in porphyric individuals.
The decreased activity of uroporphyrinogen synthetase results in secondary derepression of hepatic ALA-synthetase and over-production of the porphyrin precursors, ALA and porphobilinogen (Table 1.5, Fig. 1.16)(305). The increased urinary excretion of these substances is the main biochemical feature of AIP (305).

Thus it can be seen that prolonged administration of fluroxene produces a biochemical picture which closely resembles AIP, and therefore chronic fluroxene treatment of animals could possibly be used as a model system for studying AIP. This would be an extremely useful model system, since it provides a mammalian system for study in vivo, rather than in an in vitro system, such as chick embryo, which may be of dubious relevance to mammals. Furthermore, at present, no good experimental model exists for the study of AIP (cf. Table 1.6), and therefore chronic fluroxene treatment of animals could prove to be a new and valuable model system for the study of AIP. Experiments are currently being performed overseas with a view to establishing chronic fluroxene treatment as a

model system for human AIP.

Recently, a model system has been established in experimental animals whereby it is possible to reproduce experimentally the sensitivity of the human acute porphyric patient to lipid-soluble drugs (Section 1.8.2.) (228). Animals given a relatively small dose of DDC exhibit an experimental porphyria which closely resembles an acute hepatic porphyria (Variegate Porphyria (Section 1.7)) in its latent state, since these rats become very sensitive to drugs that can exacerbate the metabolic disorder, changing the biochemical picture to that typical of the human acute attack of porphyria (228, 306). Thus DDC treated rats have proved useful in assessing the porphyrogenicity of drugs and whether they might be potentially hazardous to the human who carries acute porphyria in the latent state (306, 307). Anderson (307) has stated that all chemicals and drugs which have been demonstrated to induce ALA-synthetase in these rats, should be avoided in patients with acute porphyria.

Since fluroxene was found to stimulate ALA-synthetase in DDC treated rats (Table 4.18), it could be regarded as being potentially hazardous for use in porphyric patients. Furthermore, in agreement with these results, Moore (309) has listed fluroxene as a drug to be avoided by patients with acute hepatic porphyria.

5.2. TRICHLOROETHYLENE

The results of previous experiments have shown that the anaesthetic agent, trichloroethylene, degrades the haem moiety of hepatic microsomal cytochrome P-450 *in vivo* and *in vitro*[+] (Section 1.3.3) (99, 100, 107).

[+] Since trichloroethylene does not contain any antioxidant, and EDTA (an inhibitor of lipid peroxidation) is present in all experiments performed *in vitro*, the observed trichloroethylene mediated degradation of cytochrome P-450 does not appear to arise from lipid peroxidation.

In agreement with the above results, intraperitoneal administration of trichloroethylene to Wistar rats was found to decrease the levels of hepatic microsomal cytochrome P-450 in vivo (Table 4.37). Furthermore, this degradation reaction appeared to affect the haem moiety of cytochrome P-450 since the losses of microsomal haem were equivalent to the losses of cytochrome P-450 (in nmoles per mg of microsomal protein) after trichloroethylene treatment of uninduced and phenobarbital induced Wistar rats (Table 4.37). Thus the trichloroethylene mediated degradation of cytochrome P-450 appeared to be similar to the fluroxene (and AIA) mediated degradation of cytochrome P-450 in vivo (cf. Sections 1.3.1 , 1.8.1.3 , 4.1.1). However, unlike fluroxene and AIA, it is not known whether trichloroethylene forms an adduct with the haem moiety of cytochrome P-450.

Since the fluroxene and AIA mediated degradations of cytochrome P-450 were thought to be associated with the pronounced effects of these chemicals on hepatic haem metabolism (Sections 1.8.1.3 , 4.1 , 5.1) it was anticipated that trichloroethylene treatment would also affect these processes in vivo. However, no change in ALA-synthetase activity and little or no change in urinary haem precursor levels were observed after acute trichloroethylene treatment in vivo (Sections 4.11.2) (Table 4.38). Acute trichloroethylene treatment did cause a significant increase in hepatic haem oxygenase activity in vivo (Table 4.37). These results are surprising since the degradation of the haem of hepatic microsomal cytochrome P-450 would be expected to cause a decrease in haem levels in the regulatory haem pool (Section 1.6.) which would in turn result in an increase in the activity of ALA-synthetase and an increase in haem biosynthesis with little or no change in the activity of hepatic

haem oxygenase (cf. AIA : Section 1.8.1.3). The lack of effect of trichloroethylene on hepatic ALA-synthetase activity (Section 4.11.2) may reflect the time at which this enzyme was assayed, viz. 24 hours after acute trichloroethylene treatment: The increase in urinary porphobilinogen levels after trichloroethylene treatment of phenobarbital induced rats (Table 4.38) suggests that a slight increase in the rate of haem biosynthesis did occur over the 24 hour collection period, but the increase was perhaps too small and/or too transient to cause any striking changes in haem precursor levels.

The increase in hepatic haem oxygenase activity observed after acute trichloroethylene treatment appeared to be related to the trichloroethylene mediated degradation of the haem moiety of hepatic microsomal cytochrome P-450 since tetrachloroethylene (the symmetrically substituted analogue of trichloroethylene (Section 1.2.4)) which caused only a slight decrease in hepatic cytochrome P-450 levels and no change in microsomal haem levels (Table 4.45), only slightly increased the activity of hepatic haem oxygenase (Table 4.46) and caused no change in hepatic haem biosynthesis (Tables 4.46 , 4.47).

The mechanism by which trichloroethylene mediates the increase in hepatic haem oxygenase activity is unknown. It does not appear to be associated with an increase in the levels of haem in the regulatory pool since trichloroethylene does not appear to cause the dissociation of intact haem from hepatic cytochrome P-450. On the other hand, the increase in haem oxygenase activity observed after acute trichloroethylene treatment (Table 4.37) may be due to induction of this enzyme; perhaps by a trichloroethylene haem adduct or a breakdown product of cytochrome

P-450 haem, as was suggested for the fluroxene mediated induction of hepatic haem oxygenase (Section 5.1).

In general, the effects of trichloroethylene on hepatic haem metabolism appeared to differ from the effects of fluroxene on these processes. Whereas acute fluroxene treatment caused pronounced increases in the rates of hepatic haem biosynthesis and biodegradation (Sections 4.1,5.1), acute trichloroethylene treatment appeared to only significantly affect hepatic haem biodegradation (Tables 4.37 , 4.38). The differences in the effects of these two compounds on hepatic haem metabolism may reflect different mechanisms of degradation of hepatic microsomal cytochrome P-450 by these two compounds, or the preferential degradation of different forms of cytochrome P-450 in vivo (cf. fluroxene, Section 1.3.1). Fluroxene is known to degrade the haem moiety of cytochrome P-450 to an N-modified porphyrin, formed by alkylation of the haem prosthetic group of cytochrome P-450 by an oxygen activated form of fluroxene (Section 1.3.1). On the other hand, although a similar product has been postulated to be formed during the cytochrome P-450 mediated oxidative metabolism of trichloroethylene (100), no green discolouration of the liver was observed after trichloroethylene treatment in vivo or of hepatic microsomes following incubation in the presence of trichloroethylene and generating system in vitro (personal observation). However, these observations do not exclude the possibility that a haem adduct (or green pigment) was formed as a result of the trichloroethylene mediated degradation of cytochrome P-450, but in amounts which were too small to be observed or alternatively, the lifetime of which was extremely short.

In order to clarify whether different forms of cytochrome P-450 are preferentially degraded by trichloroethylene and fluroxene *in vivo*, the effects of these compounds on activities which are relatively specific for different forms of cytochrome P-450 were assessed (Table 5.1) (132). Striking differences in the effects of the two anaesthetic agents on the different forms of cytochrome P-450 were observed (Table 5.1). Whereas fluroxene appears to preferentially degrade the major 3-methylcholanthrene-inducible form of cytochrome P-450 (as assessed by the activity of ethoxyresofurin deethylase) in phenobarbital induced rats, trichloroethylene did not appear to affect this form of cytochrome P-450 in phenobarbital induced rats *in vivo* (Table 5.1). In contrast, the activity of benzpyrene-3-hydroxylase, which is associated with multiple forms of polycyclic hydrocarbon-inducible cytochrome P-450, was decreased by trichloroethylene and not by fluroxene in phenobarbital induced rats (Table 5.1). The effects of fluroxene and trichloroethylene on the phenobarbital-inducible forms of cytochrome P-450 (as assessed by ethylmorphine demethylase and aminopyrine demethylase) were similar (Table 5.1). These results indicated that different forms of cytochrome P-450 were degraded by trichloroethylene and fluroxene *in vivo* and may account, in part, for the different effects of these two compounds on hepatic haem metabolism in experimental animals.

In contrast to the very slight effects of acute trichloroethylene treatment on hepatic haem biosynthesis, chronic trichloroethylene treatment caused a pronounced increase in the rate of hepatic haem biosynthesis

TABLE 5.1

THE EFFECTS OF TRICHLOROETHYLENE ON HEPATIC MICROSOMAL CYTOCHROME P-450 *IN VIVO*

Animals were induced with phenobarbital (80 mg/Kg for one day) and treated with one trichloroethylene injection (1 ml/kg) 24 hours later at time zero. All rats were starved immediately after phenobarbital treatment and sacrificed 1½ hours after the trichloroethylene treatment or at an equivalent time in control animals. Ethoxyresofurin deethylase activity was determined as described in Section 3.5.1.4. Benzpyrene-3-hydroxylase activity was measured as described by Prough *et al.* (310) and aminopyrine demethylase activity was assessed according to the method of Hazel (311). Each value corresponds to determinations in triplicate or more on a group of 3 rats. Reported values are percentages of activity remaining after trichloroethylene treatment. These results are compared to the results obtained after fluroxene treatment of phenobarbital induced rats *in vivo* as assessed by Bradshaw *et al.* (132).

ANAESTHETIC AGENT	CYTOCHROME P-450	BENZPYRENE-3-HYDROXYLASE	ETHOXYRESOFURIN DEETHYLASE	AMINOPYRINE DEMETHYLASE	ETHYLMORPHINE DEMETHYLASE
TRICHLOROETHYLENE	67 ± 2	80 ± 5	106 ± 4	86 ± 8	-
FLUROXENE[+]	63 ± 13	107 ± 3	49 ± 14	-	73 ± 2

[+] Results from Bradshaw *et al.* (132)

(Tables 4.39-4.44); in particular, the levels of urinary uroporphyrin and coproporphyrin were elevated 2 to 8 fold after the second through to the sixth injection of trichloroethylene (Tables 4.41 , 4.42), whereas the levels of ALA and porphobilinogen were only slightly elevated after the second trichloroethylene injection (Tables 4.39 , 4.40). These results indicate that the enzyme uroporphyrinogen decarboxylase may be inhibited by chronic trichloroethylene treatment, but that the activity of this enzyme should be determined after chronic trichloroethylene treatment in order to clarify this suggestion. The observation that the levels of the haem precursors returned to control values after the sixth injection of trichloroethylene (Tables 4.39 - 4.44) implies that any enzyme defect (if present) was either temporary, or was overcome by the large increase in haem biosynthesis precipitated by chronic trichloroethylene treatment.

The above results are in striking contrast to the effects of chronic fluroxene treatment on hepatic haem biosynthesis. Whereas chronic treatment of trichloroethylene resulted in the temporary elevation of urinary uroporphyrin and coproporphyrin, chronic fluroxene treatment caused a prolonged and striking increase in the levels of porphobilinogen and to a lesser extent ALA as a result of the fluroxene mediated inhibition of the enzyme uroporphyrinogen synthetase (Sections 4.3 , 5.1). Thus the different patterns of precursors excreted after chronic trichloroethylene and fluroxene treatments may be explained by the observation that fluroxene inhibited uroporphyrinogen synthetase, whereas trichloroethylene may inhibit, to some extent, uroporphyrinogen decarboxylase. The explanation for the different effects

of the two compounds on hepatic haem biosynthesis, viz. decreased uroporphyrinogen synthetase as opposed to an apparent decrease in uroporphyrinogen decarboxylase is at present unknown.

5.3. HALOTHANE

Previous experiments have shown that halothane causes a decrease in the levels of hepatic microsomal cytochrome P-450 *in vivo* and *in vitro* (16, 78, 139). Results presented in this thesis confirm that [1] intraperitoneal administration of halothane to Wistar rats *in vivo* and [2] incubation of halothane with hepatic microsomes from Wistar rats *in vitro*, resulted in the loss of hepatic microsomal cytochrome P-450 (Tables 4.21 , 4.24 , 4.33 , 4.35 , 4.36). Prior metabolic activation of halothane by hepatic cytochrome P-450 appeared to be required for halothane to elicit its effects on cytochrome P-450 since the incubation of hepatic microsomes and halothane *in vitro* in the absence of an NADPH-generating system did not affect the levels of cytochrome P-450 (Table 4.33) (16). It appears that the halothane mediated loss of cytochrome P-450 primarily reflects the degradation of the phenobarbital-inducible form of the enzyme since the losses of the phenobarbital-inducible form of cytochrome P-450 - as assessed by the metyrapone-ferrocytochrome P-450 complex - were identical ($P > 0.1$) to the losses of hepatic microsomal cytochrome P-450 (in nmoles per mg of microsomal protein) *in vitro* and *in vivo* (Tables 4.35 , 4.36). This was recently confirmed by de Groot *et al.* (313) using halothane plus hepatic microsomes *in vitro*.

The halothane mediated decrease of cytochrome P-450 appears to arise as a consequence of the presence of one or more reactive species produced

during the metabolism of halothane by cytochrome P-450 (cf. fluroxene (Section 5.1) and AIA (Section 1.8.1.3)). The destruction of cytochrome P-450 by halothane does not however appear to arise from lipid peroxidation since all experiments performed in vitro were in the presence of EDTA, an inhibitor of lipid peroxidation (312). Furthermore, thymol, which is the antioxidant used at 0.01% to preserve halothane, and is a potential inducer of lipid peroxidation, did not cause a measurable change in the levels of cytochrome P-450 in vitro in the presence of microsomes, and NADPH-generating system plus EDTA (cf. Section 3.3) (results not shown).

As proposed in Section 1.3.2 , the halothane mediated decrease of hepatic cytochrome P-450 appears to reflect in part the ability of halothane to degrade the haem moiety of cytochrome P-450 and in part its ability to facilitate the dissociation of haem from the active site of cytochrome P-450 (16). Our observation that the losses of microsomal cytochrome P-450 exceeded the losses of microsomal haem (in nmoles per mg of microsomal protein) after incubation of halothane with hepatic microsomes for 25-30 min in vitro (Table 4.33) was consistent with the above proposal. Furthermore, significant levels of cytochrome P-420 (Fig. 1.10) were produced after halothane treatment in vivo[+] and in vitro (Figs. 4.3 , 4.4) (Tables 4.21 , 4.24 , 4.25 , 4.34).

The halothane mediated degradation of the haem moiety of cytochrome P-450 appeared to be a more rapid process than the dissociation of the

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[+] The amount of cytochrome P-420 produced after halothane treatment in vivo is apparently too small to cause a significant difference between the losses of hepatic microsomal cytochrome P-450 and haem (Tables 4.21 , 4.24).

haem from apocytochrome P-450 *in vitro* and *in vivo*. After incubation in the presence of halothane *in vitro* the losses of microsomal cytochrome P-450 and haem (in nmoles per mg of microsomal protein) were initially equivalent but subsequently (after incubation of halothane with microsomes for 25-30 min), the loss of hepatic cytochrome P-450 exceeded the loss of microsomal haem (Table 4.33). Furthermore, significant levels of cytochrome P-420 were only produced at 5 and 10 hours after halothane treatment *in vivo* (Table 4.25) although the halothane mediated degradation of cytochrome P-450 was maximal 1 hour after halothane treatment *in vivo* (Table 4.24).

The effects of halothane on hepatic cytochrome P-450 and the significant changes in the rates of hepatic haem biosynthesis and biodegradation observed after acute halothane treatment *in vivo* appeared to be related as follows:

At or prior to 1 hour after acute halothane treatment *in vivo*, halothane caused a significant and equivalent decrease in the levels of hepatic microsomal cytochrome P-450 and haem (Tables 4.24 , 4.36). As proposed above, the initial effect of halothane on hepatic microsomal cytochrome P-450 appears to be degradation of the haem moiety of the enzyme, which would be expected to result in a decrease in the levels of haem in the central haem pool which regulates the activities of hepatic ALA-synthetase and haem oxygenase (Section 1.6). Therefore the levels of ALA-synthetase would be anticipated to be initially increased after acute halothane treatment *in vivo*, as was observed (Table 4.26). Furthermore, in agreement with this proposal, a smaller increase in the activity of hepatic haem oxygenase was observed at 1 hour than at 5 and 10 hours after acute halothane treatment (Table 4.26).

At some time between 1 and 5 hours after acute halothane treatment *in vivo*, halothane appeared to facilitate the dissociation of the haem from apo-cytochrome P-450 (as indicated by the significant increase in cytochrome P-420 at 5 hours after acute halothane treatment (Table 4.25)). This effect together with the increase in ALA-synthetase activity would cause an increase in the levels of free haem in the regulatory haem pool, and as a result, the activity of ALA-synthetase decreased to control levels and below (Table 4.26), whereas the activity of hepatic haem oxygenase was increased at 5 and 10 hours after acute halothane treatment *in vivo* (Table 4.26).

The above proposals are consistent with the results obtained 24 hours after acute halothane treatment (Tables 4.21, 4.22, 4.23). Firstly, the levels of hepatic microsomal cytochrome P-450 and haem remain depressed at 24 hours, as seen at earlier times, viz. 1, 5 and 10 hours after halothane treatment (Tables 4.21 and 4.24). Secondly, the small or negligible effect of acute halothane treatment on the levels of urinary haem precursors (Table 4.23) was consistent with the relatively short time period ($\leq$ 4 hours) over which the transient increase in the activity of ALA-synthetase was maintained (Table 4.22 , 4.26). Finally, the activity of hepatic haem oxygenase remained elevated at 24 hours, as at 5 and 10 hours after acute halothane treatment (Tables 4.21 , 4.26).

The effects of acute halothane treatment on hepatic haem metabolism thus appeared to be consistent with the proposal that the halothane mediated decrease in cytochrome P-450 initially reflected the degradation of the haem of cytochrome P-450 followed by the dissociation of the holoenzyme. In order to further assess the proposed multiple effects

of acute halothane treatment on hepatic cytochrome P-450 and hepatic haem metabolism the levels of the enzyme, tryptophan pyrrolase, were determined after acute halothane treatment in vivo (Table 5.2).

Tryptophan pyrrolase is a haem containing enzyme whose haem saturation levels are thought to be closely associated with the amount of haem in the regulatory haem pool (314-316). The haem saturation ratio of tryptophan pyrrolase was determined at the times corresponding to the previous measurements of the levels of cytochrome P-450, haem oxygenase and ALA-synthetase, viz. at 0, 1 and 5 hours after acute halothane treatment. At 1 hour after acute halothane treatment, no change in the haem saturation ratio of tryptophan pyrrolase was observed, whereas at 5 hours after acute halothane treatment the haem saturation ratio of tryptophan pyrrolase was strikingly increased (Table 5.2). These results provide strong support for the proposed effects of halothane on cytochrome P-450 and haem metabolism. The increase in the haem saturation ratio of tryptophan pyrrolase 5 hours after acute halothane treatment is indicative of an increase in haem levels in the regulatory haem pool, presumably as a result of the halothane mediated dissociation of cytochrome P-450. Furthermore, no change in the haem saturation ratio of tryptophan pyrrolase was observed 1 hour after acute halothane treatment which supported the proposal that the dissociation of cytochrome P-450 by halothane occurred between 1 and 5 hours after acute halothane treatment. Although a decrease in the haem saturation ratio of tryptophan pyrrolase was expected 1 hour after acute halothane treatment due to the halothane mediated degradation of cytochrome P-450, the decrease in hepatic haem levels associated with this event may have occurred prior to this time point or have been too transient to be observed by this assay.

TABLE 5.2

THE EFFECTS OF ACUTE HALOTHANE TREATMENT ON THE HAEM SATURATION RATIO OF HEPATIC TRYPTOPHAN PYRROLASE IN PHENOBARBITAL INDUCED RATS IN VIVO

Rats were induced with phenobarbital (80 mg/kg for one day) and treated or not with one halothane injection (1 ml/kg) 24 hours later, at time zero. All rats were starved immediately after phenobarbital treatment and sacrificed at the times after the halothane treatment indicated or at an equivalent time in controls. The haem saturation ratio of tryptophan pyrrolase was determined as described by Badaway (314). Each value corresponds to determinations in duplicate or more on a group of 3 rats.

HOURS AFTER HALOTHANE INJECTION	DOSAGE OF HALOTHANE ml/kg	HSR* OF TRYPTOPHAN PYRROLASE	CYTOCHROME P-450	
			nmoles/mg mic. protein	% Relative to controls
0	0	0.10±0.04	1.82±0.03	
1	1.0	0.14±0.05	1.44±0.07	79
5	0	0.45±0.04	2.06±0.05	
5	1.0	1.26±0.03	1.73±0.08	84

Abbreviations: mic., microsomal; HSR, haem saturation ratio

* HSR represents holoenzyme activity/apoenzyme activity.

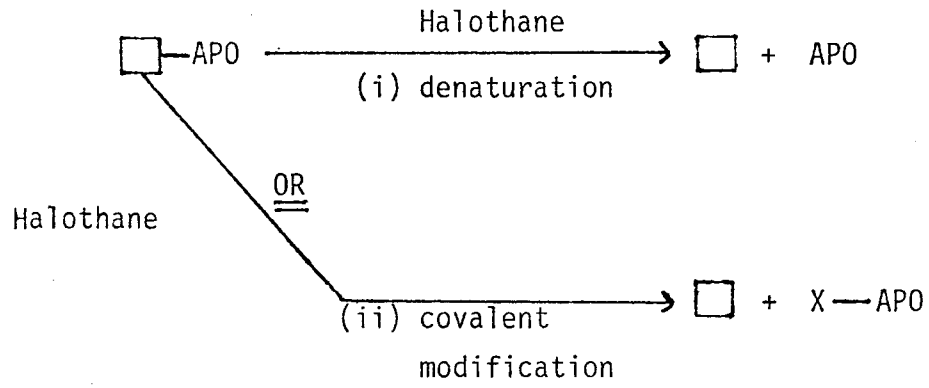
From the above results (Table 5.2) and the results presented in Section 4.7, it appeared that acute halothane treatment initially (ca. 0 - 1 hour after halothane treatment) mimics AIA treatment of animals in that it induced the activity of ALA-synthetase presumably as a consequence of its ability to degrade the haem moiety of cytochrome P-450 (cf. AIA, Section 1.8.1.3). Subsequently (ca. 1-5 hours after halothane treatment) the effects of halothane on hepatic haem metabolism were similar to the effects of carbon disulphide on these processes (Section 1.9), viz. halothane appeared to facilitate the dissociation of the haem of cytochrome P-450 and thereby increased the levels of haem in the regulatory pool, resulting in the increased activity of hepatic haem oxygenase and the normal or decreased activity of ALA-synthetase.

Whereas the effects of carbon disulphide on hepatic cytochrome P-450 and on hepatic haem metabolism are associated with the carbon disulphide mediated covalent modification of the apoprotein moiety of cytochrome P-450 (Section 1.9), the effect of halothane on apocytochrome P-450 is unknown and may reflect either denaturation or covalent modifications of apocytochrome P-450 (Fig. 5.1).

Chronic halothane treatment was found to cause the rates of hepatic haem metabolism to oscillate as a function of time (Tables 4.27 - 4.32). The activity of ALA-synthetase and the levels of the urinary haem precursors were increased after two injections of halothane (Tables 4.28 - 4.32) which may result from the following circumstances: The ability of a single injection of halothane to decrease the levels of hepatic microsomal cytochrome P-450 and to increase the activity of hepatic haem oxygenase (Table 4.21) plus, perhaps, the reinforcement or extension

Fig. 5.1

The proposed effects of halothane on the apoprotein moiety of hepatic microsomal cytochrome P-450



☐—APO	represents unchanged cytochrome P-450
☐	represents the haem moiety of cytochrome P-450
APO	represents the apoprotein moiety of cytochrome P-450
X-APO	represents a covalent modification of apocytochrome P-450

of this effect by a second injection of halothane (Table 4.27) would be expected to deplete haem from the regulatory haem pool. This process would lead to the observed increased activity of hepatic ALA-synthetase and consequently to increased flux through the haem biosynthetic pathway, as evidenced by the observed increases in the levels of urinary haem precursors (Tables 4.28 - 4.31). At a subsequent time (viz. after the sixth injection of halothane), the increased flux through the haem biosynthetic pathway would be anticipated to increase the levels of haem in the regulatory haem pool to normal levels and to result in the return of the activities of hepatic ALA-synthetase and haem oxygenase to control levels (Tables 4.27 , 4.32). After the tenth injection of

halothane, the cycle would appear to be initiated once again, since increased flux through the haem biosynthetic pathway (viz. elevated levels of urinary ALA and porphobilinogen) was observed (Tables 4.28, 4.29).

The levels of hepatic microsomal cytochrome P-450 and haem remained low throughout the duration of the experiment presumably as a result of the continued destruction of cytochrome P-450 by halothane (Table 4.27).

The effects of chronic halothane treatment on haem biosynthesis and biodegradation were in striking contrast to the effects of chronic fluroxene treatment on these processes. Chronic fluroxene treatment resulted in a decrease in the activity of hepatic uroporphyrinogen synthetase and increases in the activity of hepatic ALA-synthetase and in the levels of urinary ALA and porphobilinogen, thus inducing an experimental porphyria which closely resembled Acute Intermittent Porphyria (Section 5.1).

In contrast, chronic halothane treatment resulted in cyclical changes in haem metabolism and as a result did not produce consistent striking effects on hepatic haem metabolism nor did it induce a state of porphyria in experimental animals. These contrasting results are proposed to arise as a consequence of the differing effects of fluroxene and halothane on hepatic cytochrome P-450 (cf. Sections 1.3.1 , 1.3.2 , 5.1 , 5.3).

5.4. CONCLUSION

The destruction of cytochrome P-450 by various xenobiotics plays an important role in their ability to affect hepatic haem metabolism. However, compounds which destroy hepatic microsomal cytochrome P-450 by different mechanisms appear to affect hepatic haem metabolism in different ways, as follows:

Compounds such as fluroxene and trichloroethylene which destroy the haem moiety of cytochrome P-450 cause increases in the rates of hepatic haem biosynthesis and/or haem biodegradation (Sections 5.1 , 5.2). Specific differences in the degradation of cytochrome P-450 haem by these two compounds appear to be responsible for the different effects of these compounds on hepatic haem metabolism; a single injection of fluroxene causes the induction of both hepatic haem biosynthesis and biodegradation (Section 5.1), whereas acute trichloroethylene administration results in an increase in hepatic haem biodegradation but little or no change in hepatic haem biosynthesis (Section 5.2).

In contrast, chemicals such as halothane, which appear to cause both the degradation and the dissociation of cytochrome P-450 result in cyclical changes in hepatic haem biosynthesis and haem biodegradation in a manner which corresponds to the differing effects of this compound on cytochrome P-450 (Section 5.3).

Although the effects of acute administration of these compounds on hepatic microsomal cytochrome P-450 appear to play an important role in determining their effects on hepatic haem metabolism, the effects of chronic administration of the anaesthetic agents on hepatic haem biosynthesis appear to result from the effects of these compounds on enzymes

of the haem biosynthetic pathway and/or their effects on cytochrome P-450. Whereas fluroxene and trichloroethylene appeared to inhibit specific enzymes of the haem biosynthetic pathway, viz. uroporphyrinogen synthetase and uroporphyrinogen decarboxylase respectively. Halothane did not apparently affect a particular enzyme of haem biosynthesis. Since a block in the haem biosynthetic pathway results in the induction of ALA-synthetase and a buildup of haem precursors before the enzymatic block, administration of these and other chemicals which inhibit a particular enzyme of haem biosynthesis, results in the induction of a state of experimental porphyria in animals which may resemble a particular type of human genetic porphyria. For example, fluroxene administration which resulted in the inhibition of uroporphyrinogen synthetase and an increase in ALA-synthetase activity as well as increased levels of urinary ALA and porphobilinogen, induced an experimental porphyria in animals which closely resembled Acute Intermittent Porphyria (AIP) (Section 5.1). Similarly, chronic trichloroethylene treatment appeared to affect the haem biosynthetic enzyme uroporphyrinogen decarboxylase since increased levels of urinary ALA, porphobilinogen and particularly uroporphyrin and coproporphyrin were observed. These results suggested that chronic trichloroethylene induced an experimental porphyria in animals which resembled human Porphyria Cutanea Tarda (PCT) (Section 5.2).

In contrast, halothane did not appear to affect any particular enzyme of haem biosynthesis, but caused cyclical changes in hepatic haem metabolism. Since halothane did not produce consistent striking effects on haem metabolism, it did not induce a state similar to human genetic porphyria in experimental animals (Section 5.3).

From the results presented in this thesis, it is clear that the effects

of chemicals on hepatic microsomal cytochrome P-450 and hepatic haem metabolism are extremely varied and appear to depend on several factors which may include one or more of the following:

(a) The life-time of the drug in the body, viz. its lipid-solubility and rate of metabolism;

(b) The pathways for the metabolism of the drug, e.g. halothane is metabolized via both oxidative and reductive pathways which are both thought to produce reactive species capable of destroying cytochrome P-450 (Section 1.2.3);

(c) The various forms of cytochrome P-450 involved in the metabolism of the suicide substrates, e.g. for fluroxene, the 3-methylcholanthrene-inducible form of cytochrome P-450 appears to metabolize fluroxene slowly but to be more susceptible to degradation by fluroxene than other forms of the enzyme in rats pretreated with phenobarbital (132);

(d) The extent of degradation of cytochrome P-450 by the drug;

(e) The nature of the modification of cytochrome P-450 by the drug: i.e., is the haem and/or the apoprotein moieties modified: Is the haem moiety of cytochrome P-450 converted to green pigments viz. N-alkylated porphyrins, or is its breakdown to mono- or dipyrroles or smaller fragments enhanced.

(f) The ability of the drug to affect or not a particular enzyme of the haem biosynthetic pathway.

Obviously, in view of the complexity of the factors affecting the relationship between the degradation of hepatic cytochrome P-450 and its subsequent effects on haem metabolism, it is not possible at this time to predict the detailed effects of a drug on haem metabolism in vivo. It is, however, clear that chemicals which destroy hepatic microsomal cyto-

chrome P-450 will in general affect hepatic haem metabolism as follows: Chemicals which stimulate the dissociation of cytochrome P-450 are expected to increase hepatic haem biodegradation, whereas chemicals which degrade the haem moiety of cytochrome P-450 are expected to increase haem biosynthesis. The specific details of their effects on hepatic haem metabolism will be influenced by the variables described above.

Consistent with these general proposals is the observation that the widely used contraceptive agents, norethisterone and ethynylestradiol, which modify the haem moiety of hepatic cytochrome P-450 to green pigments (317, 318), increase hepatic haem biosynthesis (318). Furthermore, a variety of drugs including griseofulvin, morphine and the interferon inducing agents, tilorone and polyriboinosinic acid.polyribocytidylic acid, destroy hepatic microsomal cytochrome P-450 and affect hepatic haem metabolism *in vivo* (319-322).

From the evidence available in the literature, together with the results presented in this thesis, it is anticipated that chemical agents which are known to destroy cytochrome P-450 such as the widely used industrial agents bromoethane and 1,1,1-trichloroethane, cis- and trans-dichloroethylene (324), as well as compounds which contain an allyl group such as allyl bromide, allyl chloride, allyl isothiocyanate (16) and the commonly used drug, secobarbitone (323), may produce pronounced effects on hepatic haem metabolism *in vivo*.

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